PHYSICAL AND CHEMICAL INTEGRITY OF LONG LASTING INSECTICIDAL NETS (LLINs) USED IN CONTROL OF MALARIA VECTOR IN KIRINYAGA COUNTY, KENYA

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DECLARATION

This thesis is my original work and has not been presented for a degree or award in any other university.

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To everyone who helped get this report the way it is, but in particular to Simon Ngigi for his unending support and understanding, and most of all to Isaac Kangethe and James Kangethe who have given me the reason to carry on.

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ACRONYMS AND ABBREVIATIONS

ACTs	Artemisinin-based Combination Therapies
AI	Active Ingredient
APIs	Active Pharmaceutical Ingredients
DDT	Dichloro-Diphenyl-Tricloroethane
GC	Gas Chromatography
GCMS	Gas Chromatography Mass Spectrometer
HPDE	High Density Polyethylene
HPLC	High-Performance Liquid Chromatography
IRM	Integrated Resistance Management
IRS	Indoor Residual Spraying
ITNs	Insecticides Treated Nets
IVM	Integrated Vector Management
KMIS	Kenya malaria Indicator survey
LLlNs	Long Lasting Insecticides Nets
MOH	Ministry of Health
NMCP	National Malaria Control Program
PBO	Piperonyl Butoxide
pHI	Proportionate Hole Index
PHO	Public Health Office
RDT	Rapid Diagnostic Tests
SP	Sulfadoxine-Pyrimethamin
SIM	Single Ion Monitoring
WHO	World Health Organization
WHOPES	World Health Organization Pesticides Evaluation Scheme
WMR	World Malaria Report
CI	Confidence Interval
APVMA	Australian Pesticides and Veterinary Medicines Authority
ID	Internal Diameter
BPX5	5% Phenyl Polysilphenylene-siloxane.
EI	Electron Ionisation
UNICEF	United Nations Child Education Fund
IQR	Interquartile Range

ABSTRACT

The use of treated nets specifically long-lasting insecticidal nets (LLINs) has greatly reduced morbidity and mortality due to malaria. Mosquito is the vector which is responsible for malaria transmission. One of the most effective strategies of controlling the vector is the use of treated nets by majority in a community in malaria prone areas. In order to achieve a high coverage of households with the treated nets, free mass net distribution was introduced in all malaria endemic areas in Africa by the World Health Organisation. In Kenya the first free mass net distribution was carried out in the year 2006 and there after every five years. Washing of the nets causes a decline in chemical efficacy of nets. Due to this factor, both physical integrity and insecticidal concentration of distributed nets in Kirinyaga County, Kenya was assessed. The study was carried out on an area which had received treated nets from the Ministry of Health, during the 2016 mass net distribution. Most studies that have been carried out in the area focused on the rate of net survival but little is known about the physical state and chemical content of LLINs after their distribution. The objective of the study was to evaluate use, care, physical and chemical integrity of the distributed long-lasting insecticidal nets used in control of malaria in Kirinyaga County. A total of 420 households were systematically sampled with a random start, and consent to participate in the study was sought from the household heads/spouses. A structured questionnaire assessed use, care and physical integrity while chemical content of the LLINs was analysed in the laboratory by gas chromatography mass spectroscopy (GCMS). Data was analysed using Statistical Package for Social Sciences (SPSS) version 19. Eighteen months after the November 2016 nets distribution 97.9% (95% CI: 96.4% – 99.3%) of the distributed nets were still present. Regarding the net utilization, 94.1% of household heads reported sleeping under an LLIN the previous night. On visual examination of the nets, 49.9% (95% CI: 43% - 52.8%) of the nets had at least one hole. The median number of holes of any size was 2 [inter-quartile range (IQR) 1-4], with most holes located on the lower parts of a net, [median 3 (IQR 2-5)]. Of the nets with holes, only 15% had been repaired. The mean insecticidal content for baseline nets was 40.38 ± 0.86 mg/g and 9.05 ± 2.13 mg/g for α-cypermethrin and permethrin treated nets respectively. The mean concentration of sample nets was 16.29 ± 4.08 and 1.55 ± 0.41 mg/g for α-cypermethrin and permethrin treated nets respectively. Based on proportionate hole index Chi-square test results show that net physical integrity varied significantly with the manufacturer ($X_{(6, N = 389)} = 29.124$, $p < 0.05$). The proportion of households with good LLINs was 69% .There was no association between the extent of net damage and the location ($X_{(2, n = 336)} = 40.42$, $p > 0.05$). There was no notable difference in mean concentration of insecticide remaining between α-cypermethrin ($X^2 (2) = 3.83$, $p > 0.05$) and permethrin ($X^2 (2) = 4.55$, $p > 0.05$) in nets with different number of washes. The mean concentrations of α-cypermethrin and permethrin were significantly lower than the manufacturer's label claim, and a significant difference in physical integrity of LLINs from different manufacturers was observed ($X_{(6, N = 389)} = 29.124$, $p < 0.05$).

CHAPTER ONE

INTRODUCTION

1.1 Background

Malaria is a life-threatening disease, which can be prevented by controlling mosquitoes which are the major vectors of transmission (WHO, 2017).The disease is transmitted to people through bites of infected female Anopheles mosquito (UNICEF, 2020).

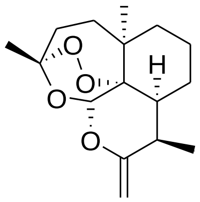
Four plasmodium species namely *P. falciparum, P. vivax, P. malariae and P. ovale* are responsible for human infection with *P. falciparum* and *P. vivax* being the most prevalent species. *P. falciparum* is the most dangerous and most common in sub Saharan Africa. *P. Knowlesi* is a fifth species which infects non-human primates but there have been reported cases of *P. Knowlesi* being found in humans in South-East Asia and the Western Pacific regions (WHO, 2019a).

Transmission factors depend on the vector, the parasite, the human host and the environment. If the mosquito life span is longer, the transmission tends to be higher, since a malaria parasite requires 7- 10 days to develop inside the mosquito into a form that is infective to humans. The genetic factor of the female mosquito and environmental conditions (temperature and humidity) are attributes that make the vector to have a long life. There are more than 400 different species of the Anopheles mosquito, of which around 40 are malaria vectors (WHO, 2019a).

World health organisation (WHO) data, shows that in 2017, an estimated 219 million cases of malaria occurred worldwide (World Malaria Report, 2017) which is a reduction compared with 239 million cases in 2010 and this is due to effective interventions which have been put in place to reduce the mortality of the disease. Malaria disease is still extremely high in Africa with over 165.9 million cases being reported (WHO, 2020). Nearly 28 million Kenyans are at risk of acquiring malaria since they live in risk zones and high malaria cases according to Kenya Malaria Indicator Survey (KMIS), (2010).

One of WHO’s strategies is to attain zero new malaria infections by the year 2030; interventions of eliminating malaria through prevention and cure are: (a) Malaria diagnosis and effective treatment, (b) Indoor residue spraying (IRS) and (c) distribution of long lasting insecticidal treated nets (LLINs).

a. With confirmed malaria cases anti-malarias are taken for treating the disease. Anti-malarial are classified as quinine related compounds, antifolates, artemisinin derivatives and non-artemisinin delivartives. Artemisinin based therapy is the recommended drug of use and the global fund quality policy is to fund only the quality-assured artemisinin-based anti-malarial drugs (Kaur *et al.*, 2015). Structure 1 is artemisinin derivartives while structure 2 is a non-artemisinin derivartive (Figure 1.1). Artemisinin combined therapies (ACTs) are used for uncomplicated malaria, while quinine with artemisinin derivatives is used for severe malaria (Bruxvoort *et al.*, 2013).



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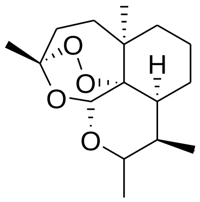

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Figure 1.1: Structures of artemisinin and non-artemisinin derivertives

b. Indoor residue spraying (IRS) is the application of a residual insecticide on surfaces, walls, eaves and ceilings of houses, where vectors are likely to come into contact with the insecticides (WHO, 2019b).

c. Universal coverage of communities with effective treated mosquito nets particularly the long-lasting insecticidal nets (LLINs) is a WHO global way of easing the malaria burden. A treated mosquito net can either repel, disable or kill mosquitoes that come into contact with it (Member States, 2016). Mosquito nets are treated with pyrethroids, the most common ones being permethrin and α-cypermethrin.

Among other strategies, a treated bed net is the most effective preventive tool for a person sleeping under it since, most mosquitoes have been reported to bite during the night (Member States, 2016). Treated nets provide both the physical barrier and insecticidal effect. In order to achieve a high coverage of households with the treated nets, programmes such as social marketing strategy on subsidized nets were extended to pregnant women and children below five years in all malaria endemic areas 2004 (Githinji *et al.*, 2010). With the introduction of free net distribution there was an increase in the number of households covered with LLINs from 6 to 79% in

Kirinyaga County within the first year of distribution (Okech *et al.*, 2008). This was a very high percentage of LLINs ownership as compared to other areas in Kenya where mass net campaigns had not started (KMIS, 2010).

The 2006 mass free net distribution made Kenya to be the focus of the international community as a learning centre for routine distribution systems, and the provision of LLINs as a complement to mass distribution and maintenance of coverage (KMIS 2010 and Hightower *et al.*, 2010). The second mass net distribution was done in the year 2010 and the third distribution was in 2016 which was the target distribution of this study.

Nets with no hole act as barriers which prevent contact between mosquito and human. Nets which are intact offer personal protection of up to seven years of use, provided the net is in good physical condition (Tami *et al*., 2004; Mutuku *et al*., 2013). Protection has been seen to disappear when a net is perforated (Irish *et al*., 2008). There is need to reduce deaths due to malaria and thus, proper follow up of the nets distributed to assess their effectiveness in malaria control is crucial.

Kirinyaga County is one of the areas with highest vector populations due to rice irrigations specifically in the Mwea region. Mwea East is predominantly rice growing whereas Mwea West’s main economic activity is horticulture. More than 74% of households in Kirinyaga own at least one net among them those which have been distributed free of cost by the public health office. However, there is little information about the efficacy and utilization of the LLINs. The study was focused on assessing the integrity, use and efficacy of nets distributed in Kirinyaga County in November 2016. There have been reports of distributed nets being misused two years after their distribution (Mutuku *et al.*, 2013, Spencer *et al.,* 2005b).

1.2 Statement of the problem

Long lasting insecticidal treated nets (LLINs) have been shown to be effective in preventing mosquitoes from having contact with the humans (WHO, 2019a). For the full benefits of LLINs to be felt by all people at risk, high coverage rates and effective nets are needed. Increasing the number of household coverage with nets in a community is of benefit to everyone (not just LLINs users) since it reduces the number of mosquitoes in the area by either repelling or killing them. In response to this, the Government of Kenya embarked on massive distribution of nets in all areas where malaria is a challenge, through the Ministry of Health.

Kirinyaga County's main economic activity is agriculture through rice irrigated farms, and this has led the county to be highly infested with mosquitoes where the farms provide a good breeding environment. Due to this high number of mosquitoes, Kirinyaga County has been earmarked to be receiving LLINs, through the free mass net distribution campaign which is done after every five years. A drop in the number of confirmed malaria parasite infections was observed in the county after the first mass net distribution (Okech *et al*., 2008).

The nets distributed in most endemic areas wear and tear at a faster rate than the expected five years before the next distribution(Girond *et al.,* 2018). There are also reports of people turning the LLINs into fencing tools to keep away birds from their gardens after only one and a half years of use (Mutuku *et al.*, 2013). Thus, there was need to monitor use, physical integrity, and levels of active chemical content of LLINs distributed in November 2016 in selected locations of Kirinyaga County, one and a half years after their distribution. According to the public health office Kirinyaga County there has been an increase in malaria cases being reported after years of zero malaria cases since the first mass net distribution in 2006.

1.3 Justification of the study

Long lasting insecticidal treated nets have been recommended by WHO as a core intervention in protecting populations from being infected with malaria. If absolute coverage (≥50% as defined by WHO) is attained, it will give a wide protection even to non-users. Proper use of LLINs determines the success of malaria disease control where LLINs are the primary prevention tools.

The levels of active ingredients in the LLINs determine the net efficiency in controlling mosquitoes. The technology behind manufacture of LLINs aims at retention of insecticidal activity even after a net is washed (Gimnig *et al.*, 2005). In addition to insecticidal loss of washed nets in laboratory studies, additional factors (routine use, heat and sunlight) same as those found when the nets are being used in the field have been shown to reduce the available insecticide on the nets (Gimnig *et al.*, 2005).

Even with mass distribution campaigns, the effectiveness of LLINs in malaria control declines with time due to nets' being stolen, population migration, wearing and tearing, and population growth. WHO recommends net distribution at an interval of three years; but due to economic constraint, distribution of nets is carried out after every five years in Kenya. Assessing the durability of these nets does not only prevent malaria resurgence but also guides in understanding the LLIN performance between different locations and populations. The results will help to support the country's program management in understanding the measure of durability for nets in routine use and serve as a guide to procurement.

1.4 Hypotheses

i. The physical integrity of LLINs and the manufacturer are not related

ii. There is no significant difference in mean concentration of permethrin and α-cypermethrin when nets are washed.

iii. The level of protection offered by the net and the number of washes are not related.

iv. There is no significance difference in chemical efficacy of baseline nets.

1.5 Objectives of the study

1.5.1 General objective

To determine the physical integrity, chemical concentration and chemical retention of long-lasting insecticidal nets (LLINs) used in the control of malaria vector in Kirinyaga County, Kenya.

1.5.2 Specific objectives

i. To evaluate usage, care and physical status of bednets from different manufacturers by administering a questionnaire.

ii. To evaluate regeneration and retention of the insecticides in baseline LLINs washed in the laboratory using Gas Chromatography - Mass Spectrometry.
iii. To determine the levels of permethrin and α-cypermethrin in LLINs used in Kirinyaga County using Gas Chromatography - Mass Spectrometry.
iv. To determine the efficacy of bednets distributed in Kirinyaga County and with different number of washes.

1.6 Scope

The study focused on assessing chemical potency, physical integrity, care and use of mosquito nets distributed in Kirinyaga County. Permethrin and α-cypermethrin were the only insecticides used in net treatment in the study area and thus the only ones considered in the study. The research area has a population density of 246 persons per square kilometre and a total area of 581km^2. The target population included all the people who received mosquito nets from the Ministry of Health in November 2016. The study was conducted between 30th April and 10th May 2018 in two sub-counties of Kirinyaga County (Mwea East and Mwea West).

1.7 Limitations

The study assessed nets that were only distributed by the Ministry of Health in November of 2016 and the name of the household head who received the net was in the net distribution master register. The study did not assess the "last time" the nets were washed, a potential limiting factor because it could not be ascertained whether complete regeneration of the insecticide had occurred prior to analysis.

CHAPTER TWO

LITERATURE REVIEW

2.1 Malaria burden

Malaria is an infectious disease which affects millions of people globally, it is a public health burden with up to 40% of its budget consumed in malaria treatment each year (Sinclair, 2017). Public health emphasis has shifted from curative to preventive health care (KMIS, 2010). In 2014, 82% of the total money from international sources in Africa bought commodities used in malaria control. Of the commodities bought LLINs consumed 63% of the total spending, ACTs 25%, rapid diagnostic kits 9%, and indoor residual spraying 3% (WHO, 2015a). More deaths occur in sub-Saharan Africa than any other part of the world; Democratic Republic of Congo and Nigeria when combined contribute more than 35% of global deaths due to malaria (WHO, 2015a).

2.2 Epidemiology of malaria in Kenya

Parasite prevalence in Kenya is less than 5 percent in more than 60 percent of Kenyan land mass which represents 78 percent of habitable land (KMIS, 2010). Malaria cases in Kenya are more pronounced in some regions (zones) with climate, temperature and altitude increasing the risk. The four epidemiological zones are;

Highland areas – in these areas parasite prevalence is low and malaria transmission is limited. Seasons favour transmission, which varies year to year and climatic conditions of around 18°C favours sustainability. But in case of epidemic malaria cases are severe, more than those experienced in regions where malaria is a common disease.

Endemic areas – these are areas with altitude ranging between 0 to 1300 metres. They are the Lake Victoria, Western Kenya, parts of Mt. Kenya (including Kirinyaga) and coastal regions. These areas have malaria transmission throughout the year.

Seasonal transmission areas – these are the North and South-Eastern parts of the country, where malaria transmission occurs in rainy seasons. The areas are characterised by high temperatures and water pools which provide breeding sites for the malaria vector.

Low risk areas - These are the Kenya highlands including Nairobi. The area has very low temperatures which are not favourable for a complete malaria life cycle.

2.2.1 Malaria in Kirinyaga

In Kenya and around the world, major efforts have been made to reduce and eliminate malaria in line with Millennium Development Goals and Kenya's Vision 2030. The Ministry of Health, through the National Malaria Control Programme (NMCP), has implemented policies and strategies in the fight against malaria. Key interventions include the provision of long-lasting insecticidal nets, intermittent preventive treatment for pregnant women, and prompt diagnosis and effective treatment of all malaria cases.

In Kirinyaga, malaria is predominantly a local disease, and the main malaria vectors are *Anopheles gambiae sensu stricto, An. Arabiensis,* and *An. Funestus,* (Zhou et al, 2006). *Anopheles Gambiae* generally increases in density after the start of the long rains, while *An. Funestus* density is seen to vary in direct proportion to the proximity of permanent breeding grounds. A considerable part of Kirinyaga is under rice irrigation which makes it ideal for malaria vectors.

The reduction of malaria cases in the referral hospital in Kirinyaga over successive years is a reflection of the effectiveness of malaria control measures implemented by this community (Okech *et al*., 2008). Between 1990 and 1995 malaria accounted for 13% of the total deaths reported in Mwea division. However, according to Okech and others only 4% of the households report malaria related deaths (Okech *et al*., 2008).

2.3 Control of the malaria vector

Progress towards malaria control is attributed to control interventions employed, which includes; indoor residual spraying (IRS), long-lasting insecticidal nets (LLINs), and disease management with artemisinin-based combination therapy (ACT). Mosquito control is directed at reducing the levels of transmission, and directly connected to strengthened health systems. Globally malaria 17% of communicable diseases (WHO, 2019b).

2.3.1 Mosquito-proofing of water storage containers

Malaria disease is a heavy burden to the public health sector and environmental malaria control is a major intervention. Containers used for water storage are designed to prevent mosquitoes from laying eggs on water surfaces, while tight fitting lids on containers used for water harvesting from roofs are used in order to keep mosquitoes away. When portable lids are used, they should be replaced every time they are removed. Larval habitats like water bearing containers and drains should be drained to ensure they do not host stagnant water becoming breeding grounds for mosquitoes (Member States, 2016).

2.3.2 Individual and household protection

Individual protection in homes and in the community is a key intervention in malaria control. Since mosquitoes are known to attack at night, people should wear clothing that does not expose their skin during night hours when mosquitoes are likely to bite (Member States, 2016). Repellents are applied to exposed skin or to clothing. When there is indoor biting insecticide aerosol products, mosquito coils or other insecticide vaporizers are recommended for use. Household windows, door screens and air-conditioning also reduce biting.

2.4 Chemical control of malaria vector

2.4.1 Adulticides

Chemical control method targets adult vectors and it’s aimed at reducing the number of mosquitoes. Chemicals used as adulticides are applied on the surface or space spraying done in vast areas (WHO, 2019b).

2.4.2 Indoor residue spraying (IRS)

Malaria vector enters into the household walls, ceilings or animal sheds to rest, IRS is applied on these surfaces (walls and ceilings) (WHO, 2019b). When a mosquito lands on the surface which has been sprayed, the lethal dose of the insecticide is absorbed through the skin causing death. This drastically reduces mosquito vector population in the vicinity and a community effect is achieved.

IRS was extensively used during the global malaria eradication campaign carried out in 1955 to 1969 (WHO, 2015a), but it was found to have negative environmental impact due to dichlorodiphenyltrichloroethane (DDT) used persistence in the air. In Kenya IRS is practiced only in epidemic prone counties and in the lake endemic region.

2.4.3 Space spraying

When adult mosquitoes are targeted outdoor insecticidal spraying (space spraying) is carried out. Space spraying is a reserve of public health and pest control as an emergency control to suppress an ongoing epidemic. WHO recommends space spraying as an emergency response to malaria epidemics, due to the high cost. Non-residual insecticide is used which degrades quickly and does not persist in the environment (WHO, 2019b).

2.5 Insecticide-treated nets

In 1992, WHO gave new guidelines on prevention and control of malaria disease. The renewed measure was the use of treated bed nets known as insecticide treated nets (ITNs) (Lengeler, 2004). Treated nets became the main strategies of reducing deaths caused by malaria and between 2010 and 2015, the percentage of people sleeping under a treated net increased by 23% globally, which led to a drastic decline in malaria disease and deaths due to the disease (WHO, 2013a; 2016).

ITNs are treated with pyrethroid, a chemical barrier, which reinforces the physical barrier. Treated nets do not only reduce the number of times a mosquito bites human beings but also increases the efficacy of the nets. Insecticides either repel or kill mosquitoes that land on it. This greatly reduces mosquito population, and if majority of the people use the nets, a community is protected since few mosquitoes will be present.

Currently WHO recommends mosquito nets that are treated with pyrethroid insecticide (World malaria report, 2017). The effect of the insecticide is that, it protects the user and also prevents the mosquitoes from flying somewhere else to bite (Skovmand, 2010). There are two types of ITNs that are available in the market:

i. Conventionally treated nets. A conventionally treated net is a mosquito net which during manufacture is dipped in an insecticide.

ii. Long-lasting insecticidal nets (LLINs). LLINs are mosquito nets which are treated at the manufacturing unit. They are meant to retain their efficacy against mosquitoes for a minimum of 3 years.

2.6 Long lasting insecticide treated nets (LLINs) manufacture

During LLINs manufacture a durable insecticide formulation is topically applied onto the netting made by multifilament polyester yarn and mixing in the polymer, producing either high density polyethylene (HPDE) or polypropylene (PP). After spinning the polymer into fibres, the fibres are converted into nettings. The fibre acts as a reserve of continuous release on to the surface of the nets where it can easily be into contact with the mosquito (Smith *et al.*, 2018). Due to the binding of the insecticide within the fibres, the nets efficacy is better than conventionally treated nets (UNICEF, 2020).

Pyrethroid is the recommended class of insecticide for LLINs treatment (KMIS, 2010). The recommended types of LLINs are;

1. Pyrethroid-only nets; this can be done to both conventional and non conventional nets which have been treated at the factory, where insecticide is thoroughly mixed with the fibres.
2. Pyrethroid-PBO nets; they contain an additional molecule (Piperonyl Butoxide) in addition to the pyrethroid insecticide. A pyrethroid-PBO net has an added advantage since mosquitoes have not shown resistance to it.

2.6. Long lasting insecticide treated nets mode of distribution

The World Health Organization recommends LLINs as a core intervention for use in protecting populations at risk of being infected with malaria. Mass net distribution was started in all malaria endemic areas, which targeted children under five years and pregnant women and there after universal coverage to the general population in all malaria endemic areas.

The Kenya National Malaria Control Program (NMCP) was tasked with the distribution, and one of its major roles is to carry out campaigns and distribution of the nets after every five years. Universal coverage to all people in a community with LLINs has over taken targeted coverage previously advocated during net distribution. The distributed nets have been shown to wear faster, with major causes of net damage being regular use and rodents (Spencer *et al.*, 2005a).

2.6.1 Antenatal and child health clinic distribution

In this method distribution is focused on expectant mothers and children between the age of zero and five years since they are the vulnerable groups in the community. Distribution of the nets is through the use of already established welfare clinics for pregnant mothers and immunization clinics, which is done in collaboration with local health facilities (WHO, 2019a).

2.6.2 Mass campaign nets distribution

Mass distribution of LLINs, is carried out to populations in areas deemed as high-risk zones, where distribution is to every person within the defined zones. The universal coverage aims at achieving a net for every bed in a household which is assumed to be shared by two people. Mass campaigns achieve high and equitable coverage quickly and efficiently. In 2006, mass net distribution was started in Kenya and this greatly increased the number of households with LLINs (Githinji *et al.*, 2010).

2.6.3 Commercial net markets

Retailing of subsidized treated nets in already existing retail shops has been shown to be an effective way of net coverage (Githinji *et al.*, 2010). Social marketing is favored since donor funding is not sustainable to provide bed nets free of charge. The uptake of nets from social marketing is more pronounced in urban areas than in rural areas; since more people are able to purchase the treated nets. In a rural set up ground water is clean enough to provide good breeding sites for mosquitoes and the buying power in rural areas is low which means increased risk of malaria disease.

2.6.4 Public sector channels

Public sector channels method of distribution makes use of vouchers or coupons that are given to the members of the public. Using a voucher, the recipient obtains a net either free or at a subsidized cost through participating retail outlets. It has been reported that public sector channels have been able to deliver more nets than any other mechanisms (WHO, 2019b).

2.6.5 School distribution

In communities with partial coverage of treated nets, school age children of between 5 and 15 years are targeted. Schools provide stable and well-established points of contact with families where distribution is achieved. Schools are used for replacing campaigns nets when it is seen that community coverage levels need to be boosted, or for the keep-up campaigns due to population growth, which are carried out after every two years (WHO, 2019b).

2.6.6 Occupation-related distribution channels

In areas with specific populations like big plantation farms, miners, soldiers, forest workers together with their families, distribution of the LLINs is made through the employers' workplace programmes and farmers' organizations (WHO, 2019b).

2.7. Assessing the durability of LLINs

Durability information of different types of LLINs is important where long term coverage with the LLINs is monitored. Assessing the durability of LLINs helps in estimating the time interval between campaigns and also understanding laboratory indicators that go with net qualities. Assessing the durability of nets provides a platform where messages on net handling are communicated so that users take better care of them (WHO, 2013b).

2.7.1 Elements of nets durability

2.7.1.1 Determining net survivorship

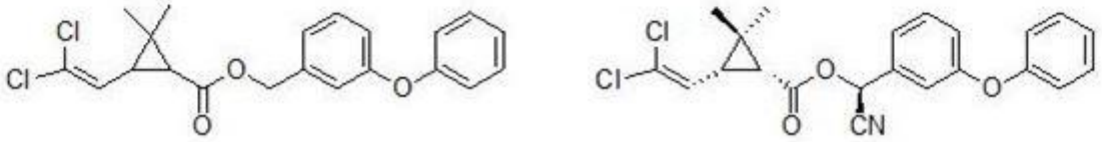
Survivorship is the ratio of the number of nets still present to the total number of distributed nets in the entire sampled area after a defined period. This is achieved by visiting all the households where nets had been distributed and confirming the net's presence (WHO, 2013b).

2.7.1.2 Physical damage of the nets

Monitoring the physical integrity (the extent of net damage) is achieved by identifying the number of holes in a net, the location, cause and size of the hole. There are generally four categories of holes defined by their diameter; 0.5 cm – 2 cm, 2cm –10 cm, 10 cm –25 cm and > 25 cm (WHO, 2013b). Nets have been reported to wear faster than expected due to damage caused by, bed frames, animals (mostly rodents), fire and age of the net. The size and number of holes in a net indicates the extent of net damage (Mutuku *et al*., 2013). Mostly mosquitoes have been shown to gain entrance in holes which are located on the upper part of the net (Lynd *et al.*, 2013).

2.7.2 Insecticidal classes of pyrethroid used in treating the nets

Earlier on pyrethroids were synthetically made to mimic pyrethrums but with advancement of research, scientists have produced pyrethroid which is more potent. Pyrethroids have very low odour and they are not toxic to mammals. There are two groups of pyrethroids; type I (where permethrin is grouped) and type II characterised by a-cyano group in their molecule which makes them more effective by way of molecular stability and insecticidal activity (α-cypermethrin is a type II pyrethroid) (Figure 2.1) (Chrustek *et al*., 2018).



3 Permethrin

4 Alphacypermethrin

Figure 2.1: Permethrin and α-cypermethrin structures

2.7.3 Working mechanism of pyrethroids

Pyrethroids are more toxic to insects than bigger animals (Chrustek *et al*., 2018). Type I pyrethroids work by changing the sodium channels' conformation while type II pyrethroids block the inhibitory neurotransmitter gamma-amino butyric acid (GABA) of the vector (Chrustek *et al*., 2018). Both types interact with the sodium channel on neuronal cell membranes thus killing the insect. Pyrethroids are not toxic to marine animals such as fish, but a few cases of toxicity have been reported in humans (Chrustek *et al*., 2018).

2.8 Analytical methods for determination of nets insecticidal content

Methods for determination and quantification of pyrethroids in LLINs are either liquid chromatography or gas chromatography using flame ionization detector

(FID), diode-array detector (DAD) or electron capture detector (GC-ECD) (Ouattara *et al*., 2013; WHO, 2015).

The methods are used for determination of only one pesticide at a time. GC-FID is suitable for baseline dosage assessment of LLINs and mostly used by manufacturers for quality control purposes (Kilian *et al*., 2008). When determining the amount of insecticide remaining in a net, gas chromatography with an electron capture detector (GC-ECD) is mostly used because of its high sensitivity (Ouattara *et al.*, 2013). GC methods are suited for separation and quantitative analysis of compounds which are volatile but thermally stable at the temperature of measurement. Pyrethroids are thermally stable compounds and thus GC method is used for analysis.

2.8.1 Chromatographic separation and interpretation in GCMS

For well separated chromatograms, different ion acquisition modes are used; full-scan, selected ion monitoring (SIM), selected reaction monitoring (SRM) and multiple reactions monitoring (MRM). Full scan is used when the analyte is unknown while SIM is used when the molecule being analyzed is known.

The response of the detector from the analyte should lie within the calibration range of standard solutions injected. Extracts with concentrations higher than the calibration curve concentrations range must be diluted before being injected. When a matrix is used in the calibration standard solutions the matrix concentration is diluted proportionately (European Commission, 2019).

2.8.2 Mass spectrometry coupled to a chromatographic column

Chromatographic column as a separation device coupled with mass spectrometry for identification is a good combination for net extract analysis. The instrument does not only give the retention time, but also mass/charge ratios (m/z) and their relative abundances in a molecule.

2.8.3 Identification using mass spectrometry technique

A reference spectrum for the analyte is generated using the same instrument and conditions used in running the standards. If there are any differences between a literature spectrum and the generated spectrum, the spectrum of the instrument is used. To maintain ion ratios (m/z) the ions with high intensity are used so that the detector is not overloaded (APVMA, 2004). In full scan, measurement is achieved by subtraction of background spectra either manually or automatically (European Commission, 2019).

2.8.4 Effects of pesticide mixtures on calibration

The detector response of individual pesticides in calibration standards with mixed pesticides is affected. In order to have uninterrupted individual pesticide response standard solutions are prepared in pure solvent and checked against calibration standard solutions of individual pesticide to confirm similarity of detector response. If the response is different, residues must be quantified using individual calibration standards by standard addition (European Commission, 2019).

2.8.5 Validation parameters and characteristics

For a quantitative method to be fit for the intended purpose, method validation is paramount. Validation characteristics considered are; linearity, range, accuracy, precision, limit of detection, limit of quantitation and selectivity (Table 2.1).

Table 2.1: Validation parameters in a method

Parameter	Measure	Criterion
Selectivity/specificity	Response in reagent blank and control samples	Deviation of back-calculated concentration from true concentration ≤ ± 20%
Linearity	Results proportional to the concentration	Test results concentration proportional to analyte in samples
Range	The interval between the upper and the lower concentration	80 – 120% of label concentration
Accuracy	The determined value of analyte should correspond to the true value.	The R value should be more than 0.995
Precision	Repeatability relative standard deviation for each spike level tested	≤ 20%
Limit of detection	Lowest concentration that is detectable	Minimum level detected
Limit of quantitation	Lowest concentration that can be analyzed	Three times the minimum level detected

Source: Australian Pesticides and Veterinary Medicines Authority (APVMA), (2004).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

Long lasting insecticides nets samples were collected from Kirinyaga County in central Kenya (0.6591° S, 37.3827° E), approximately 100 km North East of Nairobi (Figure 3.1). Kirinyaga County is at an altitude of about 1159 m above sea level with an approximate area and population density of 1478.3km^2 and 413 persons/km^2 respectively (Kenya population and housing census, 2019).

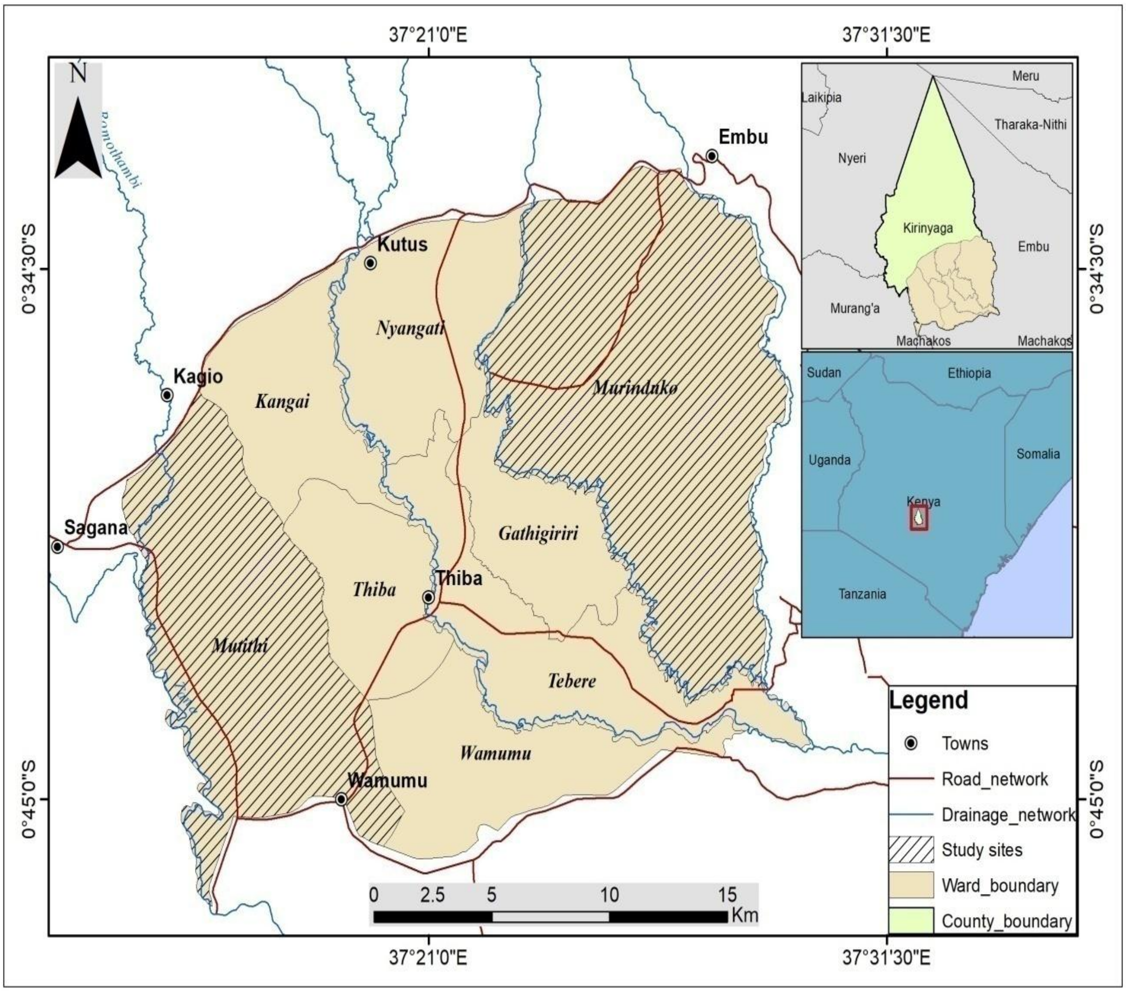


Figure 3.1: A map of Kirinyaga County - generated by ArcGIS 10.5 software

The main economic activity in the county is agriculture with rice being the main cash crop specifically in Mwea irrigation scheme. This agro-economic factor in Kirinyaga County provides breeding grounds of *Anopheles* mosquitoes that are the major vector in malaria transmission.

3.2 Sampling procedure of the study area and the households

All households within the two sub counties where LLINs had been distributed by the Ministry of Health in November 2016 during mass net campaign were sampled. The research was carried out using the guidelines and protocols given by the Scientific and Ethics Review Unit (SERU) of KEMRI with reference number KEMRI/SERU/CTMDR/037/3374 (Appendix I). The Public Health Office of Kirinyaga County was the entry point where the register of households that had received the nets was availed for this study.

The research adopted multistage sampling procedure. Stage one involved selection of two sub-counties namely Mwea East and Mwea West from the five sub-counties of Kirinyaga
County based on prevailing ecological settings (the two sub-counties have an approximate population of 142,926 people). Stage two was selection of villages from the registered 281 villages in the two sub-counties (Mwea East 141 and Mwea West 140 villages). Villages have an average of 100 households and approximate population of 500 persons. To obtain the sample size of 405 households using a uniform sample take of 15 households per village, 27 villages were required with one extra village added, to cater for missing households or where occupants had relocated to other areas. Proportionate allocation method was used to determine the

number of villages needed per sub-county and in each sub-county, systematic sampling with a random start was applied to sample required villages. The systematic sampling method was applied as it enables sampled villages across sub-county to be evenly distributed and yield good estimates for the population parameters. The final stage involved systematic sampling with a random start of eligible households within selected villages (15 households per village) and 210 households were targeted from each subcounty, and all the 420 household heads consented to participate in the study (Appendix II).

From Mutithi location in Mwea West and Murinduko location in Mwea East 420 households were selected out of which 80 households were systematically selected and a net from each household withdrawn and a replacement done (WHO, 2016). The withdrawn nets were transported to the laboratory for chemical analysis.

3.3 Sample size determination

The sample size (n) in this study was calculated from equation 3.1 with an assumption that 50% of the households selected had good nets and of good quality (Charan and Biswas, 2013). A minimum sample size of 384 was sufficient as shown in the calculation after substitutions in equation 3.1 but 420 households were sampled for this study.

$$n = \frac{z^{2}\ 1 - \alpha/2p\ (1 - p)}{d^{2}} \quad \text{…………………...................................Equation 3.1}$$

Where;

z = Standardized normal deviate corresponding to the desired level of confidence

$\underline{\alpha}$ = Type I error (Level of significance)

p = Prevalence of good quality LLINs

d = Margin of error (precision)
Applying a systematic random sampling

$$n = \frac{1.96^2\ 1- 0.5/2x0.5(1 - 0.5)}{0.05^2}$$

$$n = 384$$

3.4 Usage, care and physical damage of net

A questionnaire (Appendix III) was used to obtain the demographic data of the participants. Use and care of the nets was also assessed using the same. Net use was determined by assessing net usage the night before the survey day, while care of the net was assessed by physical observation and counting the number of holes in the net.

3.4.1 Integrity of nets

The integrity (usefulness) of the nets was evaluated using the calculated proportionate hole index (pHI), the number of holes in the net, and the area of each hole. pHI was calculated as shown in equation 3.2.

[pHI = (area/1.23 x no. of size-1 holes) + (area/1.23 x no. of size-2 holes) + (area/1.23 x no. of size-3 holes) + (area/1.23 x no. of size-4 holes)]……………….. Equation 3.2

Size one, two, three and four holes had diameters of 1.25, 6.00, 17.50 and 30.00 cm respectively (Conti 2009; WHO 2013b; 2016). Nets with a pHI value of 0 – 64, 65 – 642 and ≥ 643 were categorized as good, damaged and too torn, respectively as described by WHO (2013b). The integrity as per manufacture was evaluated, and the location of the holes (lower or upper part) recorded

3.4.2 Extraction of active ingredients from blank and sample nets

From each of the five sides of a blank net (which acted as the control) a 30 cm x 30 cm piece was cut and labeled as an individual sample. For the sample nets all the five pieces for each net were pooled together to ensure there was no bias and only one 30cm x 30cm was picked for extraction. A piece of net (10 x 10 cm) was cut from each selected 30cm x 30cm of the blank and sample nets, cut into smaller pieces and placed in 10ml glass vials containing 5ml of methanol. Chemical constituents were extracted from the nets by ultra-sonication using an ultra sonicator at room temperature for 30 min (Dieval *et al.,* 2017). The extract was filtered using polytetrafluoroethylene (PTFE) syringe filter and dilution to the appropriate concentration suitable for GCMS done.

3.4.3 Spectral analysis of samples using GCMS

A Shimadzu QP 2010-SE GCMS coupled to an auto sampler was used for the analysis Ultrapure helium gas was used as the carrier gas at a flow rate of 1ml / minute. A BPX5 non polar column, 30m; 0.25 mm ID; 0.25 µm film thickness, was used for separation. The GC was programmed as follows: 50 ℃ (1 minute); 30 ℃ /min to 300 ℃ only 1 µL of the sample were injected. Injection was done at 200 ℃ in split mode, with split ratio set to 10:1. The interface temperature was set at 280 ℃ and electron ionisation (EI) ion source was set at 200 ℃. Mass analysis was done in single ion monitoring (SIM) mode at specific retention windows. The detector voltage was: 0.96 kV + 0.00 kV with a 12.9 psi.

SIM group ions for permethrin selected were 127, 163 and 183 m/z; with 183 m/z being the quantifier ion in line with the MS library. The retention window for these ions was between 24 – 26 minutes. SIM group ions for α-cypermethrin were 127, 163 and 181; with 163 m/z being the quantifier ion. The peak area of the sample signal gave the concentration of the insecticide present when compared to the standard peak area. To test the method suitability, extraction efficiency, repeatability, accuracy and limit of detection were determined before sample injection.

All samples were analysed at the Jomo Kenyatta University of Agriculture and Technology (JKUAT) analytical chemistry laboratory. The peak area and the calibration curve results gave the amount of insecticide on a net.

3.5. Validation of the analytical method used

The method used was according to Dieval et al., (2017), “An improved extraction method for surface dosage of insecticides on treated textile fabrics”, where method suitability, extraction efficiency, repeatability, accuracy and limit of detection were determined before sample injection.

3.5.1 Selectivity/Specificity

When an instrument is able to acquire the ions of a particular analyte in presence of other compounds it shows the method selectivity (APVMA, 2004). A blank reagent (hexane) was sonicated for 30 minutes (the total extraction time) and then injected into the GCMS to check for interfering peaks from the solvent. The specificity of the method was confirmed when the detector gave signals that were defined as for the particular standard and in this method it was specified by the standards retention time (Ouattara *et al*., 2013).

3.5.2 Linearity of the detector

The linearity was checked by the consistency of the detector. Eight different concentration levels (20ppb, 50ppb, 80ppb, 100ppb, 200ppb, 300ppb, 400ppb and 500ppb) of permethrin and α-cypermethrin standards (purity > 99%) bought from Sigma-Aldrich (Taufschem, Germany) were injected twice to generate the calibration curves for permethrin and α-cypermethrin. The value of coefficient of determination (R^2) gave the linearity for each active ingredient.

3.5.3 Extraction efficiency

Extraction efficiency was determined by extracting the same 10cm x 10cm net sample using 5ml methanol and different extraction times. The extraction times were 20, 25, 30, 35 and 40 minutes. After sieving the extract the concentration for each time interval was determined using GCMS (Dieval *et al.*, 2017).

3.5.4 Precision

Precision was used to check whether the results were reproducible. The mean value of a standard solution (50ppb was used) injected repeatedly for five consecutive days was calculated, and the results reported as percentage relative standard deviation (% RSD) (APVMA, 2004).

$$\% \text{ RSD} = \frac{\text{standard deviation}}{\text{Mean}} \times 100 \qquad \text{Equation 3.3}$$

3.5.5 Limit of detection and quantification (LOD and LOQ)

LOD the lowest amount of an analyte in a sample that can be detected, was arrived at using the calibration curve plotted after running 20ppb, 50ppb, 80ppb, 100ppb, 200ppb, 300ppb, 400ppb and 500ppb of standards. From the LOD results, limit of quantification (LOQ) was calculated using the formula:

LOQ = 10/3 x LOD (Ouattara *et al.*, 2013)................................ .Equation 3.4

3.3.6 Determination of retention and regeneration time

From each of the five sides of a net, a 30cm x 30 cm piece of net material cut was placed in a 500 cm^3 beaker containing 400ml of deionised water and 200mg/l soap (pH 10-11). The beaker with the net sample was washed by placing it in a sonicator kept at 30 $^{\circ}C$ for 15 minutes. The sample was rinsed in 400ml of deionised water twice by shaking in a shaker for 15 minutes. Each net sample was dried at room temperature and stored in an incubator at 30 $^{\circ}C$ for regeneration tests; where extraction and chemical analyses using GCMS was done for seven successive days.

After washing and drying, extraction was achieved by placing the pieces of net in 5ml methanol for 30 minutes while sonicating and the extract analysed using GCMS for retention test. Extraction and chemical analysis of the active ingredients was carried out each day before the next wash, which was repeated for three days and extraction continued for four more days.

3.7 Chemicals and reagents

The solvents (methanol and hexane) and pyrethroid standards (permethrin and α-cypermethrin) of > 99% purity were bought from Sigma-Aldrich (Taufschem, Germany).

Stock solutions (100ppm) of standards (permethrin and α-cypermethrin**)** were prepared by dissolving 0.001g in 10ml hexane and stored at 4 °C (Dieval *et al.*, 2017). Serial dilutions of the stock solutions were carried out to prepare 0.01, 0.02, 0.03, 0.04, 0.05, 0.06 and 1.00 ppm working standards used to develop calibration curves.

3.7 Data analysis

The data collected was fed into a Microsoft Excel sheet and analyzed using SPSS version 19. For continuous variables, means, medians and standard deviations, were calculated and for categorical data, proportions and 95% confidence intervals were calculated. Testing for difference between grouping variable categories was performed using Chi-square (for categorical data), Student T-test and One-way Analysis of Variance (ANOVA) (for continuous normally distributed data) and Kruskal Wallis test (for continuous skewed data) depending on number of grouping variable categories.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Socio-demographic characteristics of the respondents

The questionnaire administered to the household heads showed that, out of the 420 household heads/spouses, 298 (71%) were females and 122 (29%) were males. The median age of respondents was 40 years [Interquartile Range (IQR) 30 – 51] with an age bracket of 16-90 years.

4.2 Nets physical integrity, use and care

Physical integrity was assessed by evaluating the number of holes, size and location in the net (upper side or lower side) as shown in table 4.1. The four hole-sizes (1, 2, 3 and 4) were present in the nets, of which size two (diameter = 6.00cm) was the most common with 31.5% (n = 259). In all the nets the median hole size was 2 cm [interquartile range (IQR) 1-4].

Table 4.1: Median and inter-quartile range (IQR 25-75) of holes in the nets

Size and location of holes	n	Median	IQR	Total hole area (cm^2)	Hole area cm^2 (IQR)
Size one	239	2.0	1.0 – 3.0	0.0	0.0 – 1.2
Size two	259	2.0	1.0 – 4.0	56.6	28.3 -1132.1
Size three	168	2.0	1.0 – 4.0	481.1	240.6- 962.2
Size four	154	2.0	1.0 – 4.0	1413.9	707.0-2827.8
Upper part of net	115	3.0	2.0 – 5.0		
Lower part of net	704	3.0	1.0 – 5.0		

Almost half (49.9%; 95% CI: 43.0 - 52.0) of the nets had at least one hole. More than 85 % (n = 704 out of 819) of all the holes were on the lower parts of the net as illustrated in figure 4.1. Minta *et al.* (2017) had also reported that most holes in a net are located on the lower side. The nets ability to protect the user from being biten by a mosquito could have been compromised if majority of the holes were on the top most side, since it has been shown that mostly mosquitoes enter bed nets from the upper part (Lynd *et al.*, 2013).

The number of holes and their sizes was used to calculate the proportionate hole index (pHI) of a net as a way of determining net usefulness in the households. After calculations it was found that the nets hole index ranged from 0 – 6580. Nets with pHI 0 – 65 were good nets and damaged nets had a pHI 66 – 642 while the nets with a pHI ≥ 643 were too torn nets (WHO, 2013a) (Table 4.2). Damaged nets can inhibit mosquito bites even when they are in the "damaged" condition (Randriamaherijaona *et al.*, 2017) which is partly attributed to the pyrethroids in the net fibres (Kilian *et al.*, 2008). In the study area 14.2% of the nets had a pHI of ≥ 643 and this proportion is considered to be too torn to offer any protection against mosquito bites.

Table 4.2: Net category according to Proportionate hole index (pHI)

pHI range	Net category	Number of nets	percentage
0 – 65	Good	267	68.6
66 – 642	Damaged	69	17.8
≥ 643	Too torn	53	14.2

Figure 4.1: Physical state of some nets in routine use

The major cause of net damage was found to be wear and tear due to continuous use and increased number of washes. The number of holes caused by rodents was 1.5% of the total number of nets with holes. Rodents have been found to be a big challenge when it comes to net damage (Spencer *et al.*, 2005a).

4.2.1 Comparing net physical integrity and the manufacturer

The nets distributed by the Ministry of Health in the study area had their origin from three different manufacturers as per the net labels. The manufacturers were coded as; manufacturer A, B and C. Of the total 420 households sampled only 389 nets were found within the households where 279, 84 and 26 were from manufacturers A, B and C, respectively. For the nets with holes 42% were from manufacturer A and 56 and 81% from manufacturer B and C, respectively.

Comparing the relationship between the nets physical integrity and the manufacturer Chi-square test results ($X_{(6,\ N\ =\ 389)} = 29.124$, $p < 0.05$) shows a very high relationship between the two. The statistical difference between net deterioration and the manufacturer points to underlying factors associated with defects during manufacturing; though it could not be confirmed.

4.2.1.1 Net category by location

The three net categories (good, damaged and too torn) were also used to assess the relationship between net physical damage and the location of the net, Mutithi (Mwea East) and Murinduko (Mwea West) locations. The results showed no association ($X_{(2,\ n\ =\ 336)} = 40.42$, $p > 0.05$) between the extent of net damage and the location of the net (Table 4.3).

Table 3.3: Mean hole index of bed nets by location

Net category	**Sub-location**	**n^1**	**Mean ± SE**	**%**
Good	Murinduko	124	5.25 ± 1.14	31.90
	Mutithi	143	3.47 ± 0.85	36.80
Damaged	Murinduko	39	366.91±1.90	10.00
	Mutithi	30	310.29±38.26	7.70
Too torn	Murinduko	30	1983.72±246.80	7.70
	Mutithi	23	1881.94±296.74	5.90

n^1 = number of nets, SE = standard error

4.2.2 Net usage in Kirinyaga

Net usage was high in Mwea East and Mwea West sub-counties of Kirinyaga County, with 97.9% of the nets distributed still present in the homesteads. Out of those who still had their nets, 6% reported not to have used them the previous night, despite the nets being hanged over the bed during the survey day. The findings show that net ownership does not lead to net usage.

According to findings by Okech and others (2008) high net utilization in the study area led to speedy drop of malaria incidences in the county, where sub-county health facilities reported zero positive malaria cases one year after net distribution (Okech *et al.*, 2008). This high net utilization is an indicator that people have accepted use of free nets unlike earlier reports of people disregarding them (Spencer *et al.*, 2005b; Maxwell *et al.*, 2006). The driving force behind high net usage in the area is attributed to round the year larva development, facilitated by rice irrigation farms.

Appropriate communication strategies had been designed and implemented during LLIN distribution, to educate the communities on the importance of LLINs, usage and maintenance as per WHO recommendations on individual acceptance of the nets.

Only three percent (n = 4) of the nets had been used for other purposes rather than protection from mosquito bites. Higher rates of net misuse had been reported by Mutuku *et al.* (2013) and Eisele *et al.* (2011) where in their studies they reported up to 21% of distributed nets had been found to have been mis-used. In the current study, all the mis-used nets were actually good nets as observed during physical examination (Figure 4.2). Net utilization has been found to decrease as the nets get older and the number of holes increases (Kilian *et al.,* 2008; Mutuku *et al*., 2013). Among the 35 nets not in use, 43% (n = 13) were unopened and in their original package (Table 4.4). When owners were asked what they had been using, they reported to have had excess from the previous mass net distribution. This excess ownership of bed nets was also reported by Githinji *et al*. (2010) in Western Kenya where 63% of all the unopened nets had been provided during the 2006 mass net distribution, where Kenya became the focus of international community as a learning centre for effective distribution of LLINs (KMIS, 2010).

Figure 4.2: A photograph showing misuse of a good net

Table 4.4: Reasons why the nets were not in use the previous night

Reason why net not is use	**Number of nets**	**percentage**
Gave a relative	9	2.1
Net not opened	13	3.1
Forgot to hang	5	1.2
Too torn	4	1.0
Nets in use	389	92.6

4.3 Chemical analysis

Selected ion Monitoring (SIM) method identified group ions from the selected patterns of permethrin as 127, 163 and 183 m/z while those of α-cypermethrin were 127, 163 and 181 m/z. Quantifier ions were 183 and 163 m/z for permethrin and α-cypermethrin, respectively and confirmatory ions for the same were 127 and 163 m/z for permethrin and α-cypermethrin, respectively as previously reported by European Commission (2019).

4.4 Parameters used in validation of the selected method

The parameters considered were specificity, linearity of the detector, extraction efficiency, repeatability and limit of detection (LOD) and limit of quantification ((LOQ) as described by Peters *et al.* (2007).

4.4.1 Specificity of the detector

The blank reagent (hexane) used in the extraction process confirmed absence of interfering peaks from solvents and consumables when it was run alone in the GCMS. Peaks of standards were obtained at 127, 163 and 183 m/z for permethrin and 127, 163 and 181 m/z for α-cypermethrin which corresponded with the MS library. The ability of the detector to give signals at the specified m/z showed the high specificity of the detector.

The conditions set in the chromatographic column showed well seperated peaks of permethrin and α-cypermethrin as shown in figure 4.3. The permethrin chromatogram showed two peaks with a proportion of 45/55. The first eluting peak in permethrin was R- apha a non relevant impurity of permethrin (Ouattara *et al.*, 2013). The second peak was used for quantification.

If any other positive interference was present, it was not detected between 24.67 and 27.21 minutes which were retention time for permethrin and α-cypermethrin, respectively.

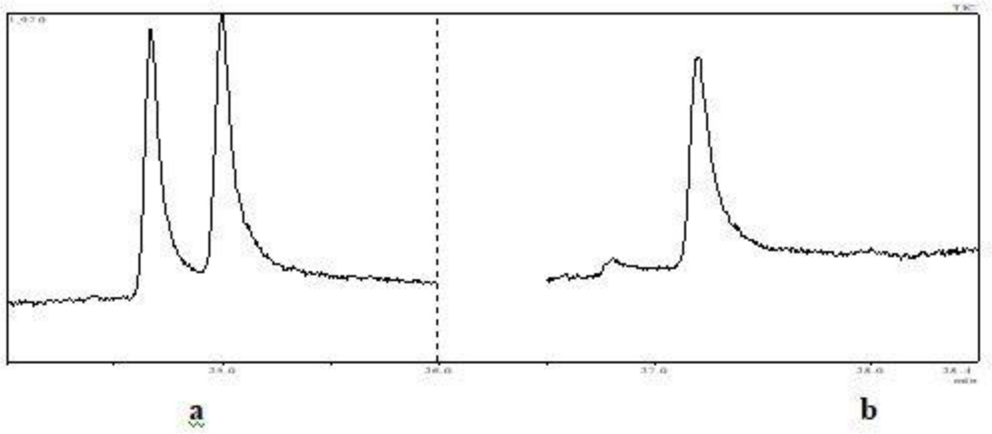


Figure 4.3: Chromatograms of (a) Permethrin and (b) α-cypermethrin

4.4.2 Calibration curves

Standard solutions of permethrin and α-cypermethrin were run in GC-MS and calibration curves were developed. Coefficients of determinations from calibration curves gave R-value of 0.997 and 0.996 for permethrin and α-cypermethrin, respectively (Figure 4.4). The values of R^2 were greater than 0.995, indicating a strong relationship between peak area and concentration of the insecticides.

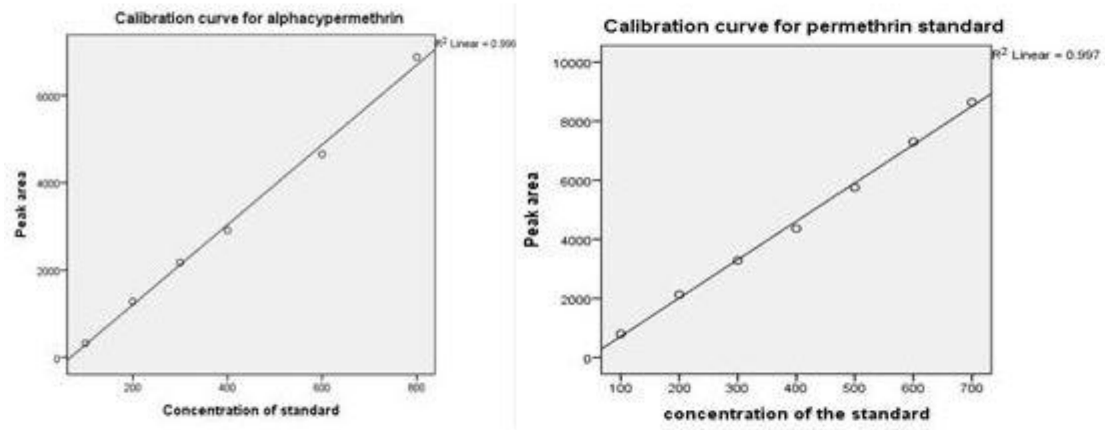


Figure 4.4: Calibration curves of permethrin and α-cypermethrin

4.4.3 Extraction efficiency of the selected method

The amounts extracted showed an optimum extraction time of 30 minutes with 19.0 and 39.1 mg/g for permethrin and α-cypermethrin, respectively being the optimum of active ingredients extracted (Table 4.5 and Appendix IV). The extraction time of 30 minutes differed significantly from the reported time of 45 minutes (Dieval *et al*., 2019) given as the optimum time for extraction of permethrin and α-cypermethrin. Unlike in this study Dieval *et al*. (2019) investigated nets that were freshly treated; in this study the nets were over one and a half years post-treatment though unused.

Table 4.5: Mean concentration of permethrin and α-cypermethrin at different extraction times

Amount extracted in mg/g		
Time (minutes)	**Permethrin mean±SE**	**α-cypermethrin mean±SE**
25	17.2±0.83	36.40±2.13
30	19.0±0.32	39.10±0.90
35	ND	ND
40	ND	ND

ND: not detected

4.4.4 Limit of detection (LOD) and limit of quantification (LOQ)

The LOD of each active ingredient was calculated from the calibration curves data chromatograms (Appendix V). The lowest standard concentration with a peak response was 0.0020 and 0.0023 mg/g for permethrin and α-cypermethrin, respectively. Three times the LOD value gave the LOQ of 0.0060 and 0.0070mg/g of permethrin and α-cypermethrin respectively.

4.4.5 Repeatability

Several chromatograms were used to assess the repeatability of the instrument (Appendix VI). The standard deviation (SD) was 10 which was within the range of those found in literature of between 9 and 11% (Kilian *et al.*, 2008).

4.5 Chemical analyses of LLINs

4.5.1 Regeneration time for baseline nets

The chemical contents of permethrin and α-cypermethrin after the first wash were significantly different from those of unwashed samples, showing loss of insecticide with washing. There was no significant difference ($p > 0.05$) in active ingredients between the third and the seventh day after washing. The results showed that complete regeneration occurred 24 hours after washing (Table 4.6). When a net is washed a certain amount of insecticide is removed. During consecutive washes more insecticide but of lesser amount is removed. This makes the decline of insecticidal content non-linear as was found after analysing net samples with different number of washes (Figure 4.5). The ability of the nets to withstand multiple washes without losing much of the insecticide makes them to last the intended five years.

Table 4.6: Mean concentrations (mg/g) of permethrin and α-cypermethrin of LLINs during regeneration

Mean concentrations of permethrin and α-cypermethrin (mg/g)									
No. of days kept			1	2	3	4	5	6	7
Permethrin	**Unwashed**		20.2mg/g						
	No. of washes	**1**	17.2	19.2	19.3	19.3	19.3	19.3	19.3
		2	16.3	17.2	17.7	17.9	17.9	17.9	17.9
		3	13.1	16.2	16.8	16.8	16.8	16.8	16.8
α-cypermethrin	**Unwashed**		39.20mg/g						
	No. of washes	**1**	35.2	38.5	38.6	38.8	38.8	38.8	38.8
		2	34.3	37.1	37.4	37.8	37.8	37.8	37.8
		3	33.8	36.4	36.8	36.9	36.9	36.9	36.9

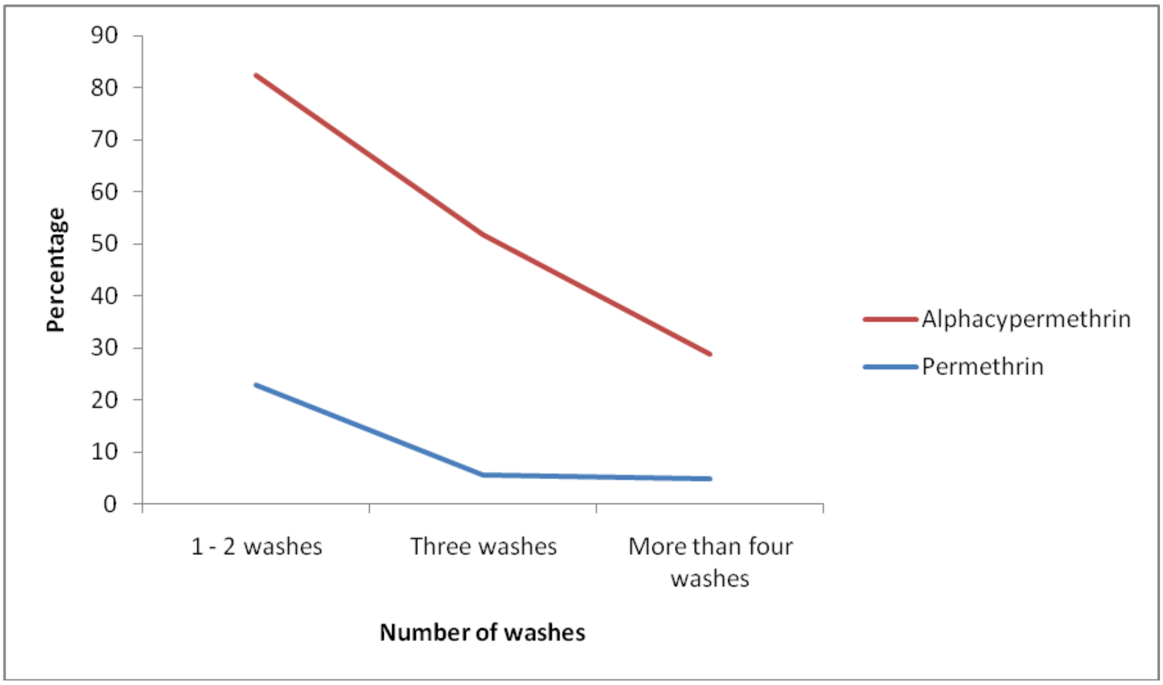


Figure 4.5: Percentage decline in concentration and non-linearity of α-cypermethrin and permethrin after net washing

4.5.2: Percentage retention of baseline nets

The active ingredients in the nets which were washed in the laboratory showed permethrin had 92 % and α-cypermethrin had 92.3 % active ingredient being retained in the nets after three washes and keeping them for seven days. The retention percent remained constant from the third day up to the seventh day after washing, showing regeneration of the insecticide had reached its optimum. The relatively equal decline in laboratory washed nets for permethrin and α-cypermethrin shows that the random distribution of nets with any of the two active ingredients would give the user the same protection against mosquito bites (Table 4.7).

Table 4.7: Mean levels (mg/g) of permethrin and α-cypermethrin retained by LLINs in baseline nets after laboratory washing

Active ingredient (AI)	No. of washes	AI (mg/g)*	Relative standard deviation	AI retention (%)
Permethrin	**unwashed**	20.20 ± 2.50		100.0
	1	19.35 ± 2.13	4.7	97.2
	2	18.60 ± 1.89	8.9	92.0
	3	18.60 ± 1.88	8.9	92.0
α-Cypermethrin	**unwashed**	39.20 ± 2.50		100.0
	1	38.80 ± 0.86	1.9	98.9
	2	37.00 ± 0.82	6.8	94.3
	3	36.20 ± 0.34	9.7	92.3

AI: active ingredient *Each value is a mean of 5 pieces of net

A washed net would protect an individual just like an unwashed net, since the bulk of insecticide resides in the sub-surface fibres (Gimnig *et al.*, 2005), and migrates (regenerates) to the surface fibres once a net is washed. Drying under the sun is the manufacturer's recommendation in order to achieve optimum regeneration. Long-lasting net technology maintains the balance of the amount of insecticide within the fibres and the amount on the surface of the fibres (Gimnig *et al.*, 2005).

4.5.3 Mean concentration of active ingredients between washed sample nets

There was no notable difference in mean insecticidal concentration in both permethrin and α-cypermethrin when compared to the number of washes a net had been subjected to; by the user ($p > 0.05$) (Table 4.8). Every time a net is washed it loses some of its insecticidal content on the surface fibres, but a replacement of the washed insecticide from the sub surface fibres to the surface fibres occurs which makes a bed net to be effective even after washing (Sreehari *et al.*, 2009).

Table 4.8: Mean concentration of permethrin and α-cypermethrin (mg/g) from sample nets

	Permethrin		**α-cypermethrin**	
Approx. Number of washes	**n^1**	**Mean ± SE**	**N^1**	**Mean**
0 – 1	8	9.08 ± 0.94	7	23.98 ± 8.95
2 -3	8	5.49 ± 0.78	9	26.80 ± 4.08
>3	11	2.08 ± 0.30	16	10.05 ± 2.56
X^2 (2) = 4.55, $p > 0.05$			X^2 (2) = 3.83, $p > 0.05$	

n^1 = number of nets

4.5.4 Analyses of α-cypermethrin and permethrin nets

Only 56.3% (n = 45) (Appendix VII and VIII) of the net samples had surface concentration of the pyrethroids above the method detection limit of 0.002 mg/g. This low number of nets with enough concentration on the surface fibres was previously reported by Smith *et al.* (2018) and Banek *et al.* (2010). Interestingly it was found that in thirteen nets permethrin and α-cypermethrin active ingredients were present in the nets (despite the manufacturers indicating only one type of insecticide had been used). There was high net-to-net variability of insecticide content within net samples, which was seen as an effect of individual use (Tami *et al.*, 2004).

Baseline nets gave a mean concentration of 40.38 ± 0.86 mg/g and 9.05 ± 2.13 mg/g of α-cypermethrin and permethrin respectively (Table 4.9). This results show that the nets did not meet the manufacturer target dosage of 241mg/g and 20 mg/g for α-cypermethrin and permethrin, respectively. This confirmed earlier reports by WHO of baseline nets not meeting the thresh hold requirement, as previously reported by Randriamaherijaona *et al.* (2017).

The within-net difference was found to have ranged from 66.6 – 19.00 mg/g for permethrin and 43.70 – 39.10 mg/g for α-cypermethrin. The distribution of permethrin active ingredients in the nets showed non-homogeneity of the insecticide. Homogeneity was observed in α-cypermethrin treated nets, though below the manufacturer target dosage. The results show only one side for both permethrin and α-cypermethrin treated nets with the target dosage specified by the manufacturer. Some sides of the baseline nets had undetectable insecticide concentration while others had more than the manufacturers target dosage (Table 4.9). Yates *et al.* (2005) similarly found nets with varying levels of chemical content within a net. Yates and others reported some sides of the net with almost three times more than the manufacturer's target dosage.

Table 4.9: Comparison between measured and target dosage of insecticide content from different sides of a baseline net

Active ingredients				
Side of a net	**Target dosage (mg/g)**	**Permethrin (mg/g)**	**α-Cypermethrin (mg/g)**	**Measured within target**
1	20.0± 2	66.6 ± 2.2^{c}		Over
2	20.0±2	0.8 ± 0.0^{a}		Under
3	20.0 ±2	ND^{a}		Under
4	20.0 ±2	15.0 ± 0.8^{b}		Under
5	20.0 ±2	19.0 ± 1.0^{b}		Within
1	241±2.5		39.1 ± 0.1^{a}	Under
2	241±2.5		43.7 ± 0.1^{b}	Under
3	241±2.5		39.4 ± 0.1^{a}	Under
4	241±2.5		40.2 ± 0.0^{a}	Under
5	241±2.5		39.2 ± 1.0^{a}	Under

ND = not detected; Mean values with the same superscript letter in a column are not significantly different ($p>0.05$).

Chemical analysis of the sample nets showed mean percentage of 17.1 % of permethrin and 40.3 % α-cypermethrin had remained (Table 4.10). . As reported by respondents “no mosquito bites us when we have covered with the nets at night” meaning that the LLINs were still effective against mosquito bite. Tungu and others in a similar study observed that concentration as low as 1.3 mg/m^2 of α-cypermethrin remaining in a net was effective in vector control (Tungu *et al*., 2016).

The major cause of insecticidal loss from a net is washing. Every time a net is washed it loses some of its insecticidal content on the surface fibres, but a replacement of the washed insecticide from the sub surface fibres to the surface fibres occurs. This makes an LLIN effective even after it has been washed (Sreehari *et al.*, 2009).

Table 4.10: Mean concentration (%) of permethrin and α-cypermethrin in sample nets

Active ingredients	Variable	n	Mean±SE	% Remaining
α-Cypermethrin	Baseline nets	20	40.38 ± 0.86	
	Sample nets	20	16.29 ± 4.08	40.3
Permethrin	Baseline nets	20	9.05 ± 2.13	
	Sample nets	20	1.55 ± 0.41	17.1

n^1 = number of nets

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

i. The proportion of households with good LLINs was 69%. There was no association between the extent of damage and the location of LLINs ($X_{(2,\, n = 336)} = 40.42$, $p > 0.05$).

ii. A washed LLIN will offer protection against mosquito bites and there is no significant difference between α-cypermethrin (X^2 (2) = 3.83, $p > 0.05$) and permethrin (X^2 (2) = 4.55, $p > 0.05$) mean concentration levels within the nets treated with the two insecticides.

iii. The results showed a relative mean percentage retention of permethrin (92%) and α-cypermethrin (92.3 %) in LLINs after controlled washing showing α-cypermethrin and permethrin treated nets have equal measure of protection before and after washing.

iv. Mean concentrations of α-cypermethrin and permethrin are significantly lower than the manufacturer's label claim, and a significant difference in physical integrity of LLINs from different manufacturers was found ($X_{(6,\, N = 389)} = 29.124$, $p < 0.05$).

5.2 Recommendations

i. The study recommends intensified awareness and behaviour change communication campaigns of malaria prevention interventions such as net washing since a washed net is still effective in vector control.

ii. Change of policy to replace the nets after three years instead of the current five years since the proportion of nets which are too torn could reverse the gains so far achieved in malaria prevention.

5.3 Suggestions for further research

i. Analysis should be done to determine the effect of heat on the integrity of the net.

ii. Further study on use of more than one active ingredient in LLINs is necessary.

iii. It has been shown that the rate of decrease of insecticides is higher within the first two washes than in consecutive washes. Studies should be done to determine why this drastic change within the first washes.

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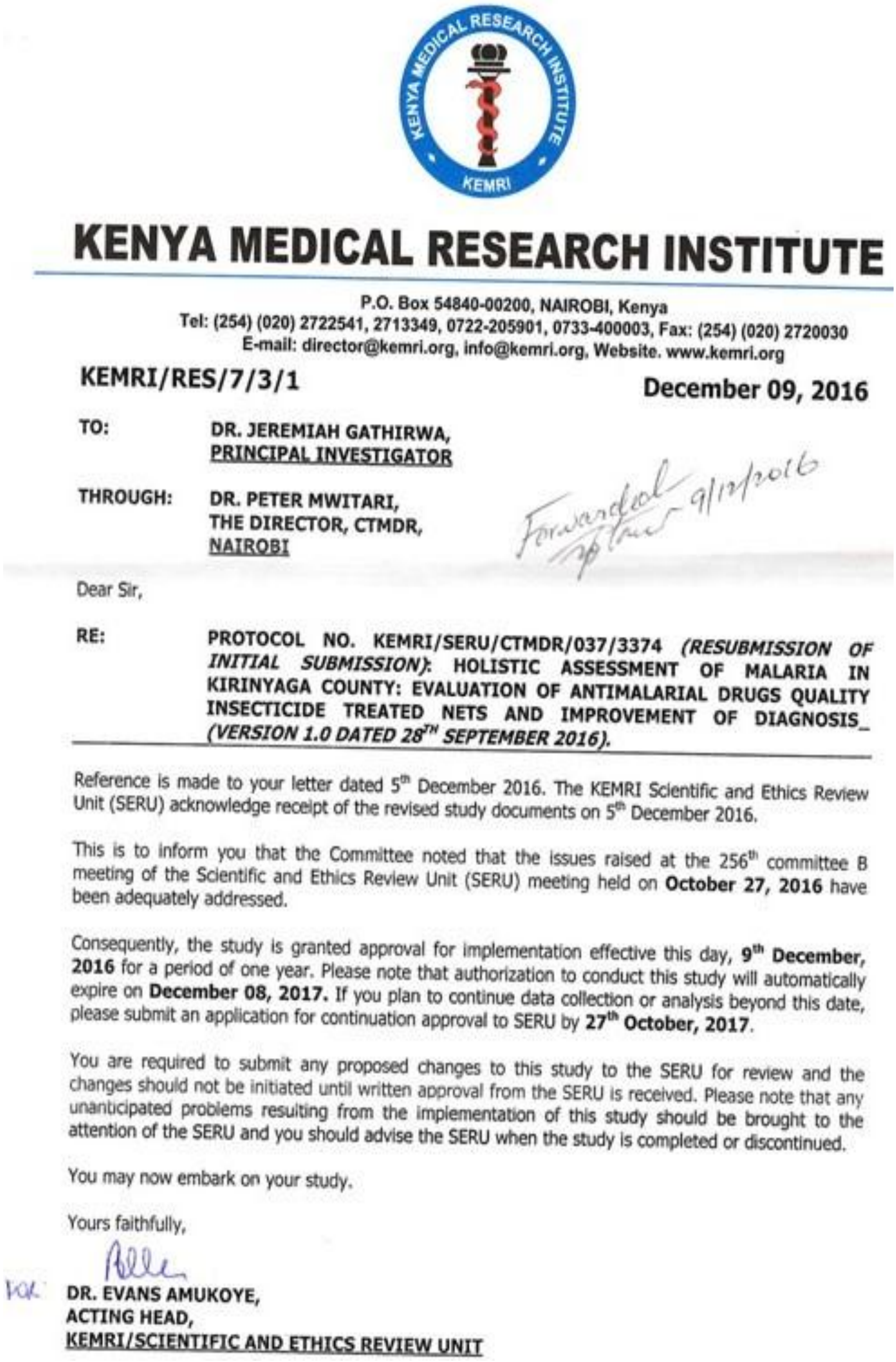
APPENDIX I: ETHICAL APPROVAL



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KEMRI/RES/7/3/1 **December 09, 2016**

TO: **DR. JEREMIAH GATHIRWA,**
PRINCIPAL INVESTIGATOR

THROUGH: **DR. PETER MWITARI,**
THE DIRECTOR, CTMDR,
NAIROBI

Forwarded 9/12/2016

Dear Sir,

RE: **PROTOCOL NO. KEMRI/SERU/CTMDR/037/3374 *(RESUBMISSION OF INITIAL SUBMISSION)*: HOLISTIC ASSESSMENT OF MALARIA IN KIRINYAGA COUNTY: EVALUATION OF ANTIMALARIAL DRUGS QUALITY INSECTICIDE TREATED NETS AND IMPROVEMENT OF DIAGNOSIS_ *(VERSION 1.0 DATED 28TH SEPTEMBER 2016)*.**

Reference is made to your letter dated 5th December 2016. The KEMRI Scientific and Ethics Review Unit (SERU) acknowledge receipt of the revised study documents on 5th December 2016.

This is to inform you that the Committee noted that the issues raised at the 256th committee B meeting of the Scientific and Ethics Review Unit (SERU) meeting held on **October 27, 2016** have been adequately addressed.

Consequently, the study is granted approval for implementation effective this day, **9th December, 2016** for a period of one year. Please note that authorization to conduct this study will automatically expire on **December 08, 2017.** If you plan to continue data collection or analysis beyond this date, please submit an application for continuation approval to SERU by **27th October, 2017**.

You are required to submit any proposed changes to this study to the SERU for review and the changes should not be initiated until written approval from the SERU is received. Please note that any unanticipated problems resulting from the implementation of this study should be brought to the attention of the SERU and you should advise the SERU when the study is completed or discontinued.

You may now embark on your study.

Yours faithfully,

DR. EVANS AMUKOYE,
ACTING HEAD,
KEMRI/SCIENTIFIC AND ETHICS REVIEW UNIT

APPENDIX II: INFORMED CONSENT FORM

PROJECT TITLE: A HOLISTIC ASSESSMENT OF MALARIA IN KIRINYAGA COUNTY: EVALUATION OF ARTEMISININ BASED DRUG RESISTANCE, QUALITY OF THE DRUGS, INSECTICIDE-TREATED NETS AND IMPROVEMENT OF DIAGNOSIS

INTRODUCTION: We are a research team from the Kenya Medical Research Institute (KEMRI). KEMRI is an institution funded by the Government of Kenya to conduct research on human health with a mission to improve it and reduce disease burden on humans. We plan to conduct a study to evaluate the insecticides treated bed nets used in the selected counties. This study has been approved by the KEMRI Scientific Ethical Review Unit (SERU). We would like to seek your permission to participate in the study.

PURPOSE OF STUDY: To assess the quality of insecticides treated bed nets if they are still effective in stopping mosquitoes form biting you at night to prevent transmission of malaria. The entire study will last for approximately 2 years. Used bed nets will be randomly selected from households to be used in evaluating the insecticides used in their treatment.

BENEFITS: You will not benefit directly from giving us your bed net, but by participating you may contribute towards the fight against malaria in your county and the country as a whole. You will receive a new bed net to replace the one collected from you.

STORAGE, TRANSPORTATION OF SAMPLES AND FURTHER STUDIES: The collected bed nets will be transported to KEMRI malaria lab in Nairobi for further studies.

I confirm that I have understood the purpose of this exercise and wish to participate in this research being conducted by the Malaria and Chemistry Units of KEMRI, Nairobi Kenya. I understand that I am free to ask any questions or to withdraw from participation at any time without penalty.

The researcher conducting this study is Ruth Nyangacha. You may ask any questions you have now, or if you have any questions later, you are encouraged to contact her through mobile telephone number: 0728710650 or email her at rnyangacha@kemri.org

If you have any questions or concerns regarding the study and would like to talk to someone other than the researcher (s), you are encouraged to contact the following:

Name of participant Signature Date

Name of Researcher Signature Date

APPENDIX III: QUESTIONNAIRE

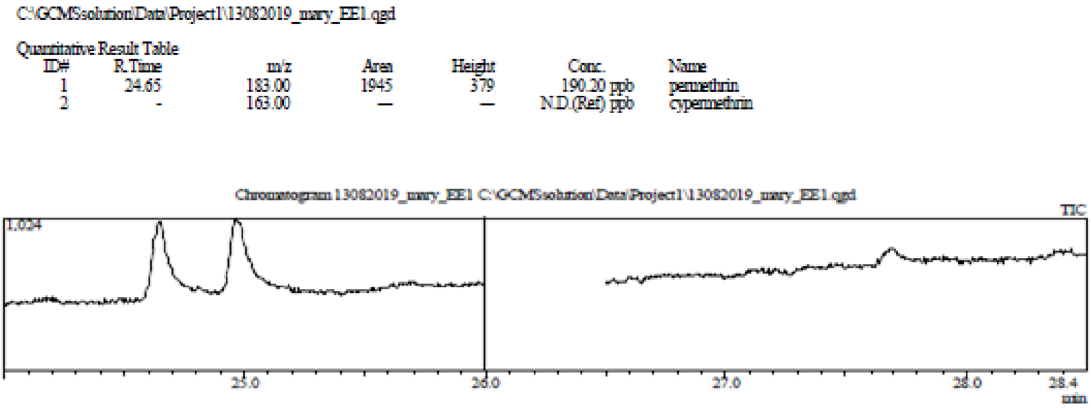
PROJECT TITLE: A HOLISTIC ASSESSMENT OF MALARIA IN KIRINYAGA COUNTY: EVALUATION OF ARTEMISININ BASED DRUG RESISTANCE, QUALITY OF THE DRUGS, INSECTICIDE-TREATED NETS AND IMPROVEMENT OF DIAGNOSIS

QUESTIONNAIRE

1	Serial Number	
2	Sample code	
3	Sampling Date	
4	Physical location	1.Sub-county
5		2. Location
6		3.Village
7	Gender	
8	Age of respondent	
9	Education level	
10	Do you have the nets supplied in 2016	
11	Was it in use last night	
12	If no why	
13	Date of manufacture	
14	Manufacturer's name	
15	Distributer	
16	Date of expiry	
17	Active pharmaceutical ingredient (API)	
18	Approximate number of washes	
19	Have you replaced the net?	
20	If yes why?	
21	What informed replacement?	
22	Physical status of the nets	
23	- Is the net intact?	
24	- Number of holes	
25	- Size of holes	
26	Has the net been repaired?	
27	If yes type of repair	
28	Cause of holes	

APPENDIX IV: EXTRACTION EFFICIENCY CHROMATOGRAM

Extraction efficiency chromatogram 1



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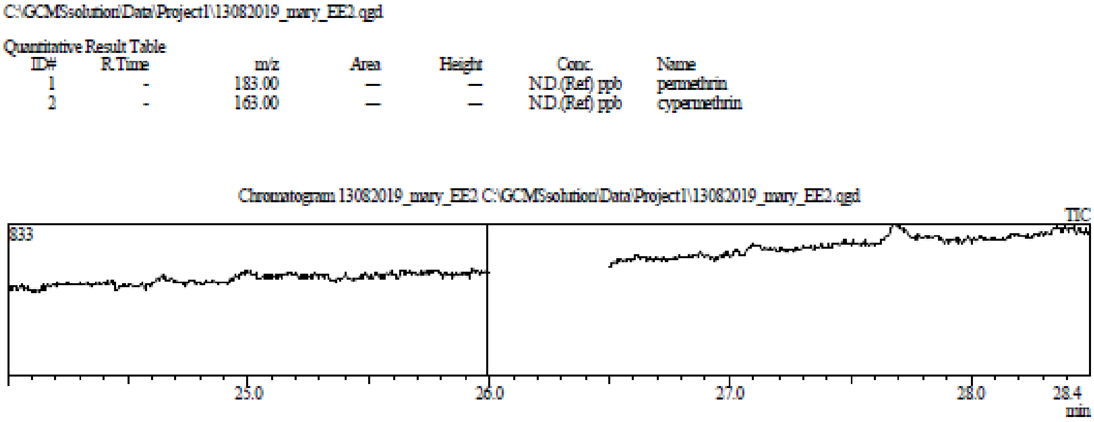
Quantitative Result Table

ID#	R.Time	m/z	Area	Height	Conc.	Name
1	24.65	183.00	1945	379	190.20 ppb	permethrin
2	-	163.00	—	—	N.D.(Ref) ppb	cypermethrin

Extraction efficiency 2



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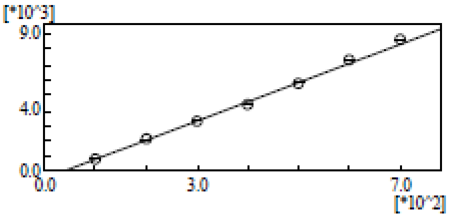
Quantitative Result Table

ID#	R.Time	m/z	Area	Height	Conc.	Name
1	-	183.00	—	—	N.D.(Ref) ppb	permethrin
2	-	163.00	—	—	N.D.(Ref) ppb	cypermethrin

APPENDIX V: CALIBRATION CURVE CHROMATOGRAMS

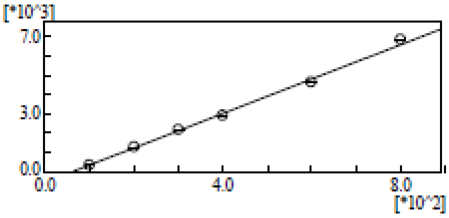
Calibration

ID#:1 Mass:183.00 Name:permethrin
f(x)=12.633755*x-457.976103
rr1=0.999127 rr2=0.998255
MeanRF:10.94 RFSD:1.43 RFRSD:13.08
CurveType:Least Square Method
ZeroThrough:Not Through
WeightedRegression:1/C2
External Standard



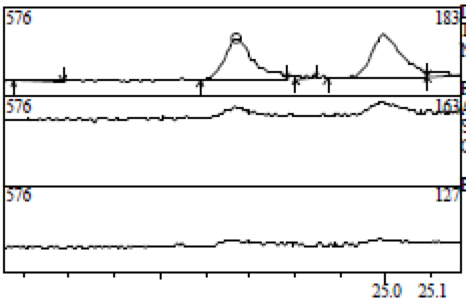
#	Conc. (ppb)	Mean Area
1	100.000	804.00
2	200.000	2130.00
3	300.000	3284.00
4	400.000	4366.00
5	500.000	5752.00
6	600.000	7297.00
7	700.000	8641.00

ID#:2 Mass:163.00 Name:cypermethrin
f(x)=8.999937*x-568.703460
rr1=0.999000 rr2=0.998001
MeanRF:6.75 RFSD:1.86 RFRSD:27.63
CurveType:Least Square Method
ZeroThrough:Not Through
WeightedRegression:1/C2
External Standard



#	Conc. (ppb)	Mean Area
1	100.000	324.00
2	200.000	1277.00
3	300.000	2172.00
4	400.000	2910.00
6	600.000	4654.00
8	800.000	6877.00

Quantitation



ID#:1 Mass:183.00
Type:Target
Name:permethrin

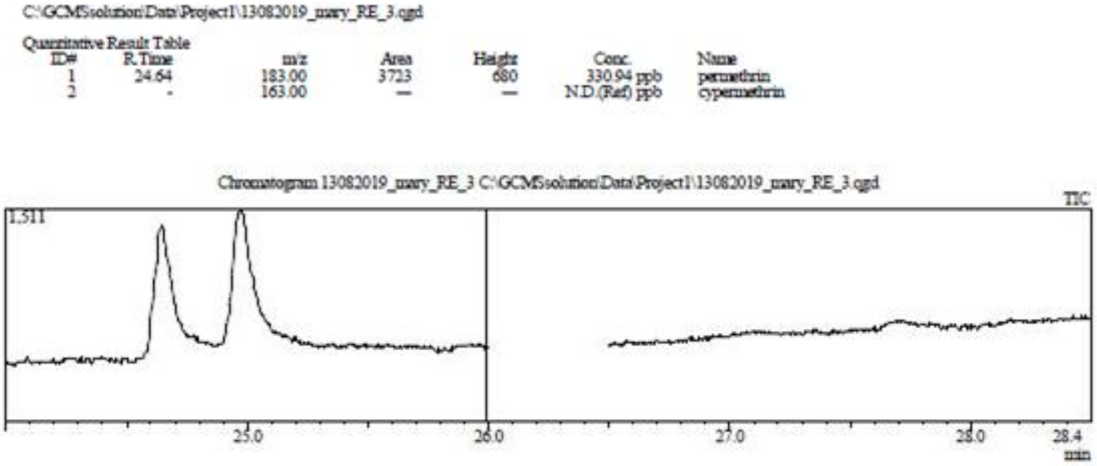
R.T:24.670
Area:1198
SI:98
Conc:131.07553ppb

Event:1:SIM

#	m/z	Intensity	Ratio
1	163.00	58	23.02
2	127.00	19	7.54

APPENDIX VI: REPEATABILITY CHROMATOGRAMS

Repeatability chromatogram 1



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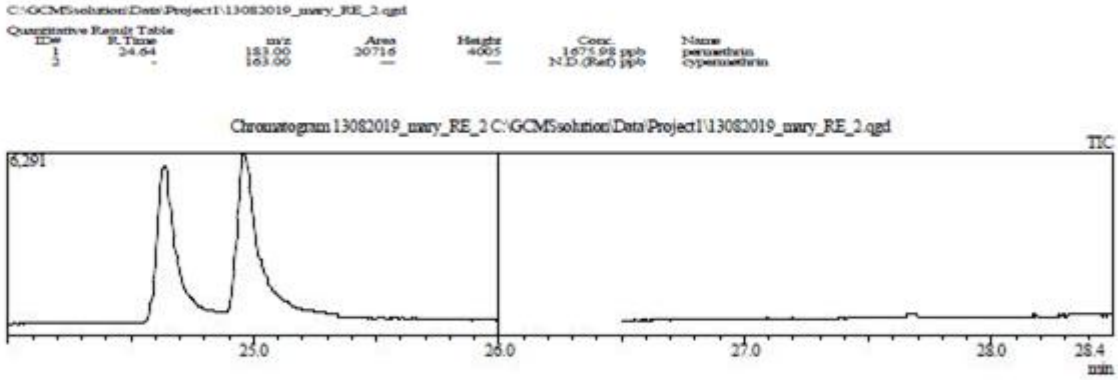
Quantitative Result Table
  ID#      R.Time          m/z       Area      Height         Conc.        Name
   1        24.64       183.00       3723         680     330.94 ppb      permethrin
   2            -       163.00         —           —   N.D.(Ref) ppb      cypermethrin
```

Chromatogram 13082019_mary_RE_3 C:\GCMSsolution\Data\Project1\13082019_mary_RE_3.qgd

Repeatability chromatogram 2



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Quantitative Result Table
  ID#      R.Time          m/z       Area      Height         Conc.        Name
   1        24.64       183.00      20716        4005    1675.98 ppb      permethrin
   2            -       163.00         —           —   N.D.(Ref) ppb      cypermethrin
```

Chromatogram 13082019_mary_RE_2 C:\GCMSsolution\Data\Project1\13082019_mary_RE_2.qgd

APPENDIX VII: PERMETHRIN TARGEATED QUANTIFIER IONS

Sample number	**Quantifier ion**	**Permethrin**		**α-cypermethrin**	
		Area	**Instrument conc. in ppb**	**Area**	**Instrument conc. in ppb**
23	183 m/z	-	-	-	-
24	183 m/z	2545	257.50	-	-
25	183 m/z	-	-	-	-
26	183 m/z	304	107.11	-	-
27	183 m/z	-	-	-	-
28	183 m/z	846	143.51	-	-
29	183 m/z	1572	192.26	-	-
30	183 m/z	-			
31	183 m/z	347	63.72	-	-
32	183 m/z	-	-	-	-
33	183 m/z	-	-	-	-
34	183 m/z	1779	177.06	-	-
35	183 m/z	-	-	-	-
36	183 m/z	-	-	-	-
37	183 m/z	299	59.92	-	-
38	183 m/z	114	94.35	-	-
39	183 m/z	367	111.34	-	-
40	183 m/z	284	58.73	-	-
41	183 m/z	-	-	-	
42	183 m/z	-	-	-	-
43	183 m/z	-	-	-	-

44	183 m/z	-	-	-	-
45	183 m/z				
46	183 m/z	-	-	-	-
47	183 m/z	64	41.32	29	66.41
48	183 m/z	-	-	29	66.41
49	183 m/z	-	-	-	-
50	183 m/z	3187	288.51		-
51	183 m/z	411	68.78	-	-
52	183 m/z	-	-	-	-
53	183 m/z	-	-	-	-
54	183 m/z	365	65.14	-	-
55	183 m/z	-	-	-	-
56	183 m/z	-	-	-	-
57	183 m/z	93	43.61	-	-
58	183 m/z	-	-	-	-
59	183 m/z	-	-	-	-
60	183 m/z	618	85.17	-	-
61	183 m/z	-	-	107	75’08
62	183 m/z	-	-	-	-
63	183 m/z	-	-	-	-

APPENDIX VIII: ALPHACYPERMETHRIN TARGETED QUANTIFIER IONS

Sample Number	**Quantifier Ion**	**Permethrin**		**α-cypermethrin**	
		Area	**Instrument conc. in ppb**	**Area**	**Instrument conc. in ppb**
64	163 m/z	-	-	71	71.08
65	163 m/z				
66	163 m/z	-	-	415	109.30
67	163 m/z	-	-	189	84.19
68	163 m/z	42.26	370.75	-	-
69	163 m/z	-	-	-	-
70	163 m/z	-	-	433	111.30
71	163 m/z	-	-	174	82.52
72	163 m/z	-	-	-	-
73	163 m/z	-	-	152	80.08
74	163 m/z	-	-	-	-
75	163 m/z	-	-	86	72.75
76	163 m/z				
77	163 m/z	950	111.45	1883	272.41
78	163 m/z	1198	131.08	850	157.63
79	163 m/z	1233	133.85	1137	189.52
80	163 m/z				
81	163 m/z	-	-	401	107.75
82	163 m/z	486	74.72	2137	300.64
83	163 m/z	32869	2637.93	-	-

84	163 m/z	3009	274.42	1709	253.08
85	163 m/z	18989	1539.29	359	103.08
86	163 m/z	12857	1053.92	896	162.75
87	163 m/z	-	-	-	-
88	163 m/z	762	95.56	433	111.30
89	163 m/z	14868	1213.10	-	-
90	163 m/z	-	-	908	164.08
91	163 m/z	-	-	1361	214.41
92	163 m/z	391	67.20	1087	183.97
93	163 m/z	-	-	233	89.08
94	163 m/z	524	77.73	2250	313.19
95	163 m/z	-	-	83	72.41
96	163 m/z	436	70.76	3058	402.97
97	163 m/z	132	46.70	3058	402.97
98	163 m/z	60	44.00	-	-
99	163 m/z	-	-	943	167.97
100	163 m/z	15356	1251.72	658	136.30
101	163 m/z	-	-	384	105.86
102	163 m/z	127	46.30	816	153.86
103	163 m/z	296	59.68	2671	359.97