OCCURRENCE OF IVERMECTIN RESIDUE IN COW MILK AFTER SUBCUTANEOUS TREATMENT AND TICKS SUSCEPTIBILITY TO ACARICIDES IN NYANDARUA COUNTY, KENYA

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DECLARATION

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

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DEDICATION

I dedicate this study to my late father, Dominic Njau Ng'ang'a, who believed in the importance of girls' education and to my mother, Rosemary Wanjiku Njau, who supported me through college education. Special dedication to my husband too, Patrick Kabui, my daughters Faith Nduta, Fiona Wanjiku, and Fidelis Wairimu, as well as to my son, Francis Njoroge, for the love, understanding, and moral support they accorded me throughout the period of the study.

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ABBREVIATIONS AND ACRONYMS

AIT:	Adult Immersion Test
ASAL:	Arid and Semi-Arid Land
CE:	Collision Energy
CID:	Collision-Induced Dissociation
ECF:	East Coast Fever
ESI:	Electron Spray Ionization
FAO:	Food and Agriculture Organization
GDP:	Gross Domestic Product
HPLC:	High-performance liquid chromatography
KNBS:	Kenya National Bureau of Statistics
LC-MS/MS:	Liquid Chromatography-Mass Spectrometer-Mass Spectrometer
MLD:	Ministry of Livestock Development
MRLs:	Maximum Residue Limits
MRM:	Multiple Reaction Monitoring
PPB	Parts per Billion
PPM	Parts per Million
RPM:	Revolution per Minute
SIM:	Selected Ion Monitoring
TBD:	Tick-Borne Disease
VDs:	Veterinary Drugs
WHO:	World Health Organization

ABSTRACT

Kenya's dairy farming has grown to meet the country's high milk demand. However, parasitic infestation, more so ticks greatly hinders production in this industry. To improve production, veterinary drugs (VDs) have been used for therapeutic and prophylactic purposes to increase production. Frequently used VDs in dairy cows include beta-agonists, anthelmintics, antibiotics, and steroid hormones. The primary chemical control methods for ticks are associated with food safety, high costs, and the emergence of resistance. There has been a great concern about the use of Ivermectin (IVM), an antiparasitic drug that is effectively used against ecto- and endoparasites in livestock. IVM accumulates and persists in animal tissues, hence restricted for use after calving of animals whose milk is meant for human consumption. Despite reports of ivermectin residues used for tick control in regions where dairy production is highly practiced. There is limited information on the presence of ivermectin residues in cow milk in Kenya. Therefore, it was important to conduct an analysis on cow milk in Nyandarua County, Kenya, to evaluate the presence of IVM residues and to ascertain whether ticks were susceptible to acaricides. One hundred and forty-nine (149) milk samples were obtained by purposeful sampling. Twenty-four (24) samples were collected post-treatment from days 0.5 to 57. Tick samples were also collected before treatment. Milk samples were analyzed using quick, easy, cheap, effective, rugged, and safe (QuEChERS), a multi-residue dispersive solid-phase extraction method coupled with Liquid Chromatography with tandem mass spectrometry (LC-MS-MS). For qualitative analysis, a retention time between 8.782 and 8.858 ($\bar{X}$= 8.362±0.002) minutes allowed the identification of the m/z 897.5 as the precursor ion and 3 product ions m/z; 897.5 > 329, 897.5 > 240, and 897.5 > 183. The method performance demonstrated a linearity of r=0.998 and 0.993 for solvent and matrix-matched calibration curves, respectively as well as a precision of 0.081 and 10.419 on retention time and recovery, respectively. The percentage recoveries for blank samples spiked with 10 ng/ml ranged from 74.977 % to 101.435 % with LOD=2.5 ng/ml and LOQ=10 ng/ml, all within satisfactory limits. The IVM was detected in milk from day 0.5 to 17 with the highest mean concentration recorded on day 2 (60.90±0.98ng/ml) post-treatment. Residue was detected in 55.84 % (n = 77) and 29.87 % (n=48) of samples taken from farms and markets, respectively. Residue levels in raw, boiled, and diluted samples indicated no significant difference in residue concentrations between raw and boiled milk samples since f_{calc} (0.011) < f_{crit} (4.196), while two-factor analysis indicated f_{cal} (11.510) > f_{crit} (2.4837) because of depletion of IVM with time. Although diluting raw samples once and twice reduced residue concentration since f_{cal} (16.446) > f_{crit} (4.196), and f_{cal} (67.240) > f_{crit} (4.196) respectively, there was no significant difference observed between single and double diluted samples since f_{cal} (1.671) < f_{crit} (2.424). Amitraz, chlorpyrifos, cypermethrin, and a mixture of (chlorpyrifos: cypermethrin) were widely used for control of ticks in this region. *R. appendiculatus* recorded resistance of between 6% and 45% to all tested acaricides, showing susceptibility. *B. decoloratus* showed 45%, 85%, 50% and 64% resistance for combined treatment, chlorpyrifos, amitraz, and cypermethrin, respectively. It was demonstrated that IVM residues persisted for 17 days in milk, and neither boiling nor dilution degraded it, and the two methods therefore may not be considered a safety precaution against consuming IVM-contaminated milk. Additionally, there was evidence of emerging resistance to cypermethrin and amitraz. Besides, the efficacy of these acaricides can be preserved with better management, ultimately resulting in the suppression of cattle ticks.

CHAPTER ONE

INTRODUCTION

1.1 Background

Human health is directly impacted by the environment, notably the type and quality of food that is available for consumption (Tilman and Clark, 2014). Due to the revolution of agro-industry, there are increased occurrences of contaminants in foods and thus, most public health agencies around the world are getting concerned over the quality of animal products (FAO, 2017). Further, quality assurance, a form of food safety management in primary production in the livestock sector is becoming increasingly important. Shortfalls in food safety, as reported in several studies in recent years, have exacerbated the situation (Grace *et al*., 2010).

During the many production stages, cows "pass" through a number of development and improvement activities including providing for the health needs of the animals, feeding, and milking. Thus, the animal healthcare system has traditionally focused on disease detection and treatment in animals. Additionally, since the 1970s, developed programs for herd fertility, herd health, and production management have been introduced extensively into dairy farming practice (Noordhuizen and Welpelo, 1996). This elicited possible sources of contaminants in animal products. Other than the production stage, contamination of animal products also emanated from the processing and packaging stages. Contamination deteriorates both the quality and quantity of milk available for human consumption (MacLachlan and Bhula, 2009). Subsequently, the existence of such residues in milk suggests that the manufacturing processes for several dairy products, such as cheese and yoghurt, have been hampered (Pérez *et al*., 2013). In comparison to other industrial branches, animal production quality refers to animal

products and/or the manufacturing process, which covers activities such as feeding, milk collection, and calf rearing (Lievaart *et al.*, 2005).

Various studies indicated pesticides, herbicides, fungicides, heavy metals, radionuclides, mycotoxins, detergents, disinfectants, dioxins (polychlorinated biphenyls and polybrominated biphenyls) and veterinary drugs (anthelmintic, antibiotic, and sulphonamide drugs, as well as somatotropin hormone) are among the major chemical pollutants in animal food products (Bushra *et al*., 2014). Some residues are left in food produced for human consumption after these contaminants enter the animal's body (Bushra *et al*., 2014; Khaniki, 2007). For many years, quality control checks in milk carried out included some classical checkpoints like the content of protein and butterfat, bacterial and somatic cell counts; mycotoxins, heavy metals, as well as the presence of antibiotic residues (Belli *et al.*, 2013; Prandini *et al*., 2009; Sospedra *et al.*, 2009; Aniello *et al*., 2006). In recent times, dairy industry has utilized quite a number of veterinary drugs intensively and for different purposes that comprised of: administering therapeutic and sub-therapeutic doses for prophylactic purposes and/or to enhance production efficiency (McEwen and Fedorka-Cray, 2002).

Ivermectin, an anthelmintic drug, has demonstrated activity against various nematode species of all stages and all economically important gastrointestinal and lung parasites. Furthermore, research has demonstrated the drug’s effectiveness against the majority of domestic animal arthropod parasites, including biting flies, ticks, penetrating mites, and parasite dipteran larvae (Canga *et al*., 2009; McKellar and Benchaoui, 1996; Chabala *et al.*, 1980; Egerton *et al.*, 1979). Although ivermectin is widely utilized in local markets, such drugs are unlabelled and no withdrawal periods are indicated.

Subsequently, the only information provided by most manufacturers in relation to such drugs is only on milk withdrawal-time caution labelled as “not to be used within 2 months of calving”. Conversely, the statement does not suggest an authentic withdrawal period as required and lacks evidence based on any residue depletion kinetics.

Similar to this, information about the depletion of ivermectin residue in milk appeared to be inadequate, and the withdrawal period has no clear definition. Consequently, additional studies have been conducted to assess whether the residues could be found and how they degrade in milk following treatment with topical and subcutaneous doses. Ivermectin was administered subcutaneously in the prescribed amounts of 0.2 $mgkg^{-1}$ bodyweight, and Toutain (1988) showed that the milk elimination half-life was 4.7 days and that there were detectable residues in milk that exceeded 5 ppb for 16 days. In other studies, ivermectin residues were detected in milk samples for 17 days following treatments of cows with a 0.58 mg/kg BW and a 5 mg/mL topical solution. The highest residue levels were found on days 3 to 4 following treatment (Escribano *et al.*, 2012; Anastasio *et al*., 2002). Inevitably, these levels are influenced by the breed, age, and environmental conditions (Chicoine *et al*., 2007). Hence, the need to evaluate the potential and levels of ivermectin in locally acquired milk samples.

Further, findings from prior trials where the same amount was administered to animals, residues were found in milk samples taken nine days after treatment of six Holstein and six Jersey cows. Comparing residues in Holstein and Jersey cows, the tested milk showed significantly higher ivermectin levels in the latter. However, milk samples were not tested beyond the 9^{th} day of treatment, hence the study could not provide a comprehensive residue depletion profile (Chicoine *et al*., 2007). Elsewhere, in Brazil,

a study of milk samples purchased from retail markets revealed that 17.8% of them were contaminated with ivermectin residues ranging from 2 to 10 ppb (Lobato *et al*., 2006). The rife occurrence of residues demonstrated that most producers in this industry were not conforming to the label's prohibition on using ivermectin in nursing dairy cows. As a result, farmers appear to have a collective influence not only on the production of animal products but also on the wellbeing of animals.

Contamination with potentially harmful compounds is presented as a serious issue because milk-based products make up such a large portion of most people's meals around the world. As a result, it is important to remember that improper veterinary medication administration might result in drug residues in edible foods. These residues may cause extreme damage to the user after intake. Some of the side effects observed are; allergic responses in hypersensitive people or the indirect creation of resistant bacteria strains by ingesting antibiotic residues. The apprehension led human health regulatory bodies around the world to establish maximum residual limits (MRLs) on residues that resulted from the use of veterinary medications and remained in tissues, milk, and eggs intended for human consumption (Kinsella *et al*., 2009). Their safety has been addressed either through the establishment of safety standards derived from "No Observed Adverse Effect Levels (NOAELs)" obtained through experiments carried out in toxicological studies, such as Acceptable Daily Intake (ADI), Tolerable Daily Intake (TDI) and/or Provisional Tolerable Weekly Intake (PTWI, for accumulating chemicals) (Fischer *et al.*, 2011). The established tolerances were a mitigation measure against harmful effects caused by residuals if ingested (Seri, 2013).

To detect veterinary drug residues in foods and tissues, quantitative and/or qualitative screening and confirmation methods using micro-biological/bioassay techniques were developed. However, because these methods yielded a semi-quantitative estimate of the "total" residues detected, they were unable to distinguish between members of the antibiotic class (Kinsella *et al*., 2009; Stolker *et al*., 2008). On the other hand, as analytical techniques advanced, extremely tiny quantities of chemical pollutants that appeared in cow milk and other products of dairy origin were found (Cheibub *et al.*, 2019; Macedo *et al.*, 2015; Furlani *et al*., 2015). The current study's analytical approach was founded on the principles of quick, easy, cheap, effective, rugged, and safe (QuEChERS) method. Acetonitrile (MeCN) was used to extract a representative fraction of the sample. Following that, a dispersive solid-phase extraction using magnesium sulfate ($MgSO_4$) and Sodium hydrogen citrate sesquihydrate was performed. Liquid chromatography-tandem mass spectrometry (LCMS/MS) was used to examine an aliquot of the supernatant after the supernatant had been cleaned.

Because such contaminants may cause bio-accumulation or bio-magnification when exposed (Zenker *et al*., 2014), chemical analysts, in collaboration with relevant bodies, are responsible and have the greatest influence in ensuring food safety, correcting abuses, and directing the proper use of these otherwise beneficial products (FAO, 2017; Mwamakamba *et al.*, 2012). As a result, the current research looked into the possibility of ivermectin residue contamination in milk from a region with a lot of dairy production, a lot of ticks, and purported resistance of ticks to most acaricides available in the market. Given that the majority of people produce and consume milk, it is imperative to ensure that widely used livestock pesticides do not however endanger the public's health.

Milk is either consumed raw or processed by different industrial or homed based processes (Claeys *et al.,* 2013). It has been reported that various milk treatment process affects its nutritional as well as chemical components (Escribano *et al.,* 2012). Boiling has been reported to reduce microbial contents and denaturation of whey proteins whereas microwaving has been demonstrated to remove microbial populations but preserve whey proteins (Tremonte *et al.,* 2014). Thermal treatment of sheep milk has been noted not to affect the stability of ivermectin (Cerkvenik *et al.*, 2002). Further, no chemical loss or bioconversion was reported in raw sheep milk after thermal treatment (Imperiale *et al.,* 2009). Much of the existing literature on the effects of processing on milk is unrelated to ivermectin residues in bovine milk. The chemical and nutritional composition of sheep milk is different from cow milk. Notably, there is limited literature regarding the effects of heating on ivermectin in cow milk globally. In fact, The Codex Alimentarius Commission indicated that the published literature on the effects of processing on chemical species in milk is insufficient (Codex Alimentarius Commission, 2023). There, more information is required to affirm the effects of heating on ivermectin residues in cow milk.

One leading factor that threatens dairy production is parasitic infestations, particularly ticks (Avcı and Filazi, 2020; Beyene, 2016). Application of anti-parasitic agents in livestock farming has been reported to increase milk production (Fthenakis *et al.,* 2000). Acaricides are among the most commonly applied drugs for parasite control (George *et al.*, 2004; De Meneghi *et al.,* 2016). Acaricides come in different classes: organophosphates, carbamates, pyrethroids, and amidines (George *et al.,* 2004). Besides, there are other chemical agents used for tick control, ivermectin (IVM). The

IVM is active against various endo and ecto-parasites as well as ticks (Piras *et al.,* 2022). It is administered orally, subcutaneously, or topically (De Meneghi *et al.,* 2016). Though effective, there is an emergency of resistant tick populations which threatens the efficacy of these agents (Neethu, 2023). Therefore, it is imperative to investigate their efficacy, otherwise, it would be a risk to production losses.

1.2 Statement of the Problem

Ivermectin has been fundamental in the management and treatment of livestock parasites. Originally IVM was developed to target helminths however its broad spectrum of activities has extended to the management of ticks in livestock (Piras *et al.,* 2022; Nava *et al.,* 2019). Notably, due to food safety concerns, the usage of IVM for the management of livestock pests and parasites is not recommended for lactating animals. IVM is persistently excreted in milk and its residues in milk meant for human consumption compromise its safety (Khalifa *et al.,* 2024). Further, there is no established withdrawal period for ivermectin among lactating animals. Besides, reported withdrawal periods in the literature are inconsistent (Escribano *et al.,* 2012). Ivermectin usage in the livestock industry in Kenya is widespread (Poché *et al.,* 2015; Mwamachi *et al.,* 1995). The drug is effective and widely affordable by many farmers (Poché *et al.,* 2015). The drug is frequently used on dairy farms for tick management in the study area, a region with a high dairy farming density in the country. Even though useful, its persistent presence in livestock muscles, viscera, fatty tissues, and milk poses hazardous risks to human health (Canga *et al*., 2009; Campbell, 2012; Alvinerie *et al*., 1993; Toutain *et al*., 1988). There is potential for off-label use and a likelihood of contamination of dairy products including milk. Unfortunately, there is no reliable and robust monitoring of ivermectin occurrence in dairy products, particularly milk, in

Kenya. In fact, most of the studies locally have focused on investigating the presence of antibiotics in milk (Ouma *et al*, 2021; Njoroge, 2020), leaving out antihelminth drugs.

Besides, there has been a resurgence of pesticide resistance globally. As a result, the use of acaricides, as a mainstream chemical control method for ticks in livestock farming has been rendered ineffective (Githaka *et al.*, 2022; Emsley, 2021; Wanzala *et al*., 2018; Abbas *et al*., 2014; Lovis *et al*., 2013; Estrada-Peña and Salman, 2013; Ferná ndez-Salas *et al*., 2012; George *et al*., 2004; De Castro, 1997; Kunz and Kemp, 1994). Unfortunately, evidence on monitoring of resistance of tick to these remedies in Nyandarua County is limited. Thus, the efficacy of these acaricides cannot be ascertained.

1.3 Justification of the study

Milk is a widely consumed beverage in Kenya. It contributes a significant proportion of food security to Kenya's populace. Food safety is paramount to protect human health. Consumption of contaminated milk products over the long run will result in hazardous harm to the larger population. Therefore, strategies are needed to carryout robust monitoring of milk safety and hygiene. This initiative will ensure the quality and safety of food products hence protect the population against potential toxicity. The present study assayed for presence and quantity of ivermectin a potential anthelminthic contaminant of dairy milk with hazardous effects to human health.

Furthermore, livestock farming in Kenya is a major source of food security. However, farmers have suffered significant financial losses as a result of livestock loss caused

by pest infestation in cattle (Valente *et al*., 2014; de Castro, 1997). Ticks are a major threat to livestock health in Kenya. Consequently, livestock loss reduces the food stability of our economy. Thus, to ensure food security it is necessary to ensure that pesticides are effective against their targets to support the livestock industry in Kenya. This study will assess whether the current conventional acaricides used against ticks are effective in this area compared to ivermectin.

1.4 Null Hypotheses

i. There is no significant difference in ivermectin residual concentrations present in milk samples collected from the market.
ii. There is no significant difference in time-dependent depletion of ivermectin residue levels in raw milk samples obtained from treated cross-bred dairy cows after subcutaneous treatment.
iii. There is no significant difference in ivermectin residue levels between raw, boiled, and diluted milk samples.
iv. There is no significant difference in the susceptibility of ticks to synthetic acaricides and ivermectin.

1.5 Objectives

1.5.1 General objective

To determine ivermectin residues in cow milk, and the susceptibility of ticks to acaricides in Nyandarua County, Kenya.

1.5.2 Specific objectives

i. To determine the presence and quantity of ivermectin residues in milk samples from farms and the market

ii. To evaluate the ivermectin residual depletion over time in raw milk samples collected from treated cows.

iii. To assess the effects of boiling and dilution on ivermectin residues present in milk samples taken from treated cows.

iv. To determine the susceptibility of ticks to conventional acaricides

1.6 Significance of the study

This study provides information against the backdrop of literature regarding safety of milk products and efficacy of acaricides and ivermectin used in control of tick in livestock farming in Nyandarua County. The data acquired from determining whether ivermectin was excreted through milk and how long it persisted in milk following subcutaneous treatment in lactating cows will inform control measures to improve the safety of livestock products. The study's findings can be used to advise on appropriate withdrawal intervals when the drug is administered subcutaneously to lactating cows and to provide information about the drug's exposure as a matter of public health safety. Additionally, determining the efficacy of current acaricides is critical to support the efforts of safe tick control in livestock farming. This study's findings can be used by appropriate authorities to guide farmers on the adoption of appropriate measures in the use of veterinary drugs.

1.7 Scope and limitations

i. The study focused solely on ivermectin residues in milk, and no other animal products were examined.

ii. Given the size of Nyandarua County, the study only covered a small portion of it.

iii. *In-vitro* resistance testing was carried out on *Rhipicephalus appendiculutus* and *Boophilus decoloratus*.

iv. The molecular pathways may be analyzed to determine the mechanism of resistance to acaricides in ticks

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

Kenya, an agricultural country, agriculture continues to be the mainstay of her economy and a fundamental economic sector in terms of domestic production. It has an estimated agricultural Gross Domestic Product (GDP) share of 33% (FAO, 2024). It is noted that 10% of Kenya's total GDP, is contributed by the livestock sub-sector. Mainly, agricultural activities in most rural households in Kenya serve as an important source of food security and employment. It is estimated that cattle production employs 50 percent of the agricultural workforce, either directly or indirectly. (Ochieng *et al*., 2016; Behnke and Muthami, 2011).

Kenya's economy is heavily reliant on livestock farming, a sub-sector dominated by ruminant species. Moreover, animal distribution is across all production systems, with an estimated contribution value of around 45% of the agricultural sector's output (Behnke, 2010). The overall number of ruminants in the 2009 census was reported to be 67 million, with 3.4 million dairy cattle, 14.1 million zebu cattle, 27.7 million goats, 17.1 million sheep, 2.9 million camels, and 1.9 million donkeys (Table 2.1) (KNBS 2010). Ruminant production contributes Ksh 318.971 billion to agricultural GDP as a whole. This represents a 150 percent increase over the official projection of Ksh 127.723 billion based on livestock figures reported in 2009. Thus, the volume of livestock product output and the amount of livestock-derived food that is available for domestic consumption are both significantly revealed by these data. According to revised estimates, milk and associated products, valued at Ksh 257.811 billion, have been the country's most valuable animal produce since 2009, thus contributing to

around 70% of all livestock's gross value to the agricultural sector (Behnke and Muthami, 2011).

Table 2.1: Kenyan livestock populations: 2009 census headcount and per cent of the total population

Animal	National MLD 2008 estimates	National 2009 population census	ASAL	Arid	Semi-arid	Highlands
Cattle	13,522,500 77%	17,467,774 100%	12,155,974 70%	6,281,354 36%	5,874,620 34%	5,311,800 30%
Sheep	9,907,300 58%	17,129,606 100%	14,954,925 87%	10,246,527 60%	4,708,398 27%	2,174,681 13%
Goats	14,478,300 52%	27,740,153 100%	25,250,865 91%	18,230,633 66%	7,020,232 25%	2,489,288 9%
Camels	1,132,500 38%	2,971,111 100%	2,968,670 100%	2,924,742 98%	43,928 1%	2,441 0%
Donkeys	786,800 43%	1,832,519 100%	1,616,522 88%	1,126,103 61%	490,419 27%	215,997 12%
Pigs	330,020 98.6%	334,689 100%	82,500 25%	1,438 1%	81,062 24%	252,189 75%
Beehives		1,842,496 100%	1,371,101 74%	286,564 16%	1,084,537 59%	471,395 26%
Chicken indigenous	29,615,000 93%	25,756,4872 81%	10,258,066 32%	1,063,276 3%	9,194,790 29%	15,498,421 49%
Chicken commercial		6,071,042 19%	1,523,983 5%	131,811 0%	1,392,172 4%	4,547,059 14%

Source: KNBS, Kenya Population, and Housing Census, Vol. II, Table 11

As the country progresses towards being a middle-income state, it should provide inhabitants with a high-quality life by 2030. However, these changing processes have several consequences, such as increased urbanization and varying income levels. Subsequently, a tremendous increase in demand for cattle and livestock by-products is envisaged (Teresiah *et al.,* 2016). As a result, the livestock industry will experience a lot of pressure by reacting to increased demand while simultaneously strengthening smallholder farmers' social-economic stability. Since the country cannot meet the enormous demand, it has to import 20-25% of some livestock products (USAID, 2014). Consequently, there has been a significant shift towards market-oriented smallholder agriculture, sometimes known as agri-business, resulting in increased use of veterinary drugs to boost production (Aytaged and Tolesa, 2004).

The dairy industry in Kenya is very liberal and thus, it has played an important economic and nutritional role to most people. Farmers, milk vendors, processors, and consumers are among those involved and make a livelihood therein (Teresiah *et al*., 2016). The milk production system in Kenya operates on two scales; large-scale and small-scale, differentiated by their operational sizes, management levels, and input usage. However, small-scale farming has dominated the industry (Omore *et al.,* 2005). Studies indicate that an estimated 1.5 million households perform small-scale dairying. It is recorded that more than 85% of milk production and 80% of the 1.8 billion litres of milk sold each year come from small-scale dairying (Staal *et al*., 2001). Primarily, Nyandarua County produces relatively high quantities of milk in comparison to other regions in Central Kenya region. This is associated with high populations of dairy cows present in the region (Muia *et al.,* 2011). However, most farmers in the region are faced

with associated production constraints, among them diseases, ticks, and helminths control practices (Maingi and Njoroge, 2010).

Utilization of chemical products in bulk production in addition to response to the expanding market for animals and animal by-products has sparked serious health concerns. The health threats do not only affect animals but also consumers either directly or indirectly (Raza and Kim, 2018). Furthermore, livestock farming has experienced a share of other challenges such as parasitic infestation. This comprises of gastrointestinal nematodes, ectoparasites like ticks, mites, and lice that cause loss of productivity in cattle. Ectoparasitic arthropods feed on, puncture, or burrow into the host's epidermal surface to eat or acquire shelter (Barré and Uilenberg, 2010; Petney *et al.*, 2007; Colebrook and Wall, 2004). To combat parasitic infestation and ease the negative effects brought about by parasites (Lopes *et al.*, 2016) and provide enough food to the growing population, farmers utilize veterinary drugs (Thornton, 2010). Other associated reasons for the use of drugs include enhanced development in animal weight, increased feed efficiency, and treating and/or avoiding infections in food-producing animals. Antibiotics, anthelmintics, beta-agonists, and steroidal hormones are given orally, by injection, or as an intramammary infusion for treating bacterial infections, expelling internal parasites, or as growth-promoting agents (Orwa *et al.*, 2017; Lees *et al.*, 2004).

Synthetic acaricides are employed in veterinary medicine to manage external parasites like ticks, whereas anthelmintic drugs are licensed to manage helminth and ectoparasitic infestations in animals (George *et al.*, 2004; Crump and Omura, 2011). Consequently, investigations have revealed that there is no single, safe anthelmintic

effective against endo and ectoparasites, owing to toxicity to both animals and people, as well as to the ecosystem (Baena-Díaz *et al*., 2018; Holter *et al.*, 1993; Sommer *et al*., 1993). Despite the fact that not all VDs contain acutely hazardous chemical substances, their residues or metabolites remain in animals and then enter the food chain. The indiscriminate use of anthelmintics available in the market is a matter of concern and needs to be addressed (Lopes *et al*., 2011).

2.1.1 Contamination routes for milk and other dairy products

Milk from ruminants has a wide variety of by-products that are considered to be a necessary component of a balanced diet, due to the significant number of micro- and macronutrients they provide to new-borns, children, and adults. (Neumann *et al*., 2002). Moreover, there are risks that quality would be compromised because of increased market demand for milk and related by-products. According to researchers, almost all domesticated female ruminants i.e. cattle which includes cows, goats, sheep, etc. are frequently treated with different drugs to boost the rate of milk production. Similarly, they are exposed to anthropogenic contaminants from other sources, providing threats when they consume contaminated grass (or hay) or drinking contaminated water. All these lead to bioaccumulation in the body, compromising milk production (Raza and Kim, 2018).

Agrochemicals (Pesticides and Veterinary medicines), environmental pollutants, and food-processing-induced chemicals are all examples of food contaminants (Di Stefano and Avellone, 2002). Biological, chemical, and physical contaminants have been identified as the most common in dairy products. Veterinary medications, heavy metals, radionuclides, mycotoxins, pesticides, nitrates, detergents, and disinfectants are among

the chemical pollutants that enter the animal body (Nag, 2010; Khaniki, 2007). Farmers also knowingly add noxious substances including formalin, hydrogen peroxide, melamine, blotting paper, baking soda, washing soda, unclean water, salt, and other pollutants to milk. Consequently, these chemicals and/or their metabolites get released with milk, contributing to serious biological effects to consumers (Fischer *et al*., 2011). Chemical additives introduced to improve on taste, texture, and shelf life of dairy foods, may have long-term serious consequences too. Pharmacokinetically, such compounds are lipophilic and accumulate in the animal fat before being transported to milk (Raza and Kim, 2018).

2.1.2 Veterinary drugs residues

For enhanced productivity, modern livestock farming systems have made extensive use of veterinary treatment in animal breeding. Veterinary pharmaceuticals, often known as veterinary medicinal products (VMPs), encompass antibiotics and sulphonamides used to treat bacterial infections, anthelmintics for expelling internal parasites from the animal body beta-agonists and steroidal hormones used as growth-promoting agents, and pesticides for pest control in animals (Lopes *et al*., 2011; Stolker and Brinkman, 2005). Research has shown that residues or their metabolites persist in animals. As a result, they enter the food chain, posing risks to animal and human health (Khaniki, 2007).

According to research, abuse of restricted drugs or the failure to observe withdrawal times before slaughtering or milking treated animals has resulted in unwanted residues in edible products (Teresiah *et al*., 2016; Souza *et al.*, 2011; Dahiya *et al.*, 2010). These medications and their metabolites have been linked to inherent toxicity and

carcinogenic effects on human health (Van Den Bogaard and Stobberingh, 2000). As a result, regulatory measures by relevant agencies must focus on the usage and distribution of veterinary drugs in animal food production systems (Schwarz *et al.*, 2001; Anadón and Martínez-Larrañaga, 1999). To preserve human food safety, major international bodies, notably the European Union and Codex, have MRLs in milk for most VDs and outlawed those that persist in food items (Orwa *et al*., 2017; Furlani *et al*., 2015; Imperiale *et al*., 2002).

Various anti-parasitic veterinary drugs that are approved for the control of helminths (parasitic worms) in animals used for food production are generally categorized as nematicides, flukicides, and endectocides. To ensure the safe use of these drugs in nursing animals, maximum residue limits (MRLs), which are the safest concentrations permitted for human exposure, have been determined with the ultimate goal of consumer safety assurance (Whelan *et al*., 2010). These MRLs are as listed in table 2.2.

It is inevitable that animals raised for food will be treated with veterinary medicine. Nonetheless, if the drug is not properly managed, the drug or its metabolites end up in animal and animal-derived products. Consequently, sensitive and specific procedures for monitoring and determining contaminants have been developed and approved (Dahiya *et al*., 2010; Whelan *et al*., 2010; Aguilera-Luiz *et al*., 2008).

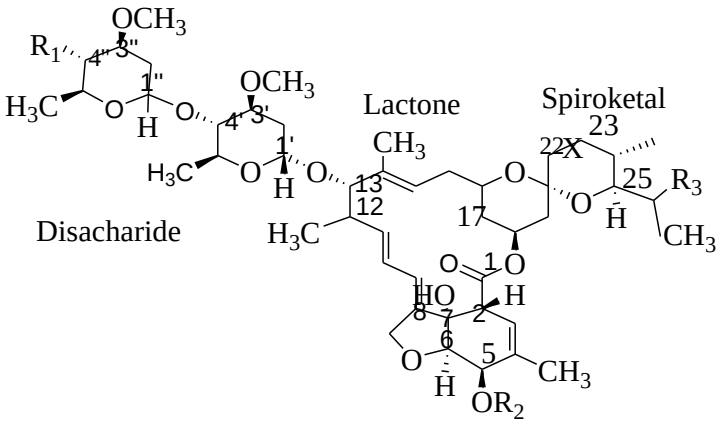
Table 2.2: Allowable MRLs of common anthelmintics

Active ingredient	Mode	Withdraw-al, days	MRL [EU]		Tolerance [US]		Codex	
			Milk	Liver	Milk	Liver	Milk	Liver
Macrocyclic lactones								
Eprinomectin	Pour on	0	20	1500	12	480	20	2000
Doramectin	Injectable	40		100		100	10	100
	Pour on	50						
Ivermectin	Injectable	35		100		100	10	100
	Pour on	49		100				
	Oral bolus	184						
Moxidectin	Injectable	36	40	100	40	200		100
	Pour on	36	40	100	40	200		
Abamectin	Injectable	42		20				
Benzimidazoles								
Fenbendazole			10	500			100	500
Fenbendazole-sulfoxide			10	500	600	800	100	500
Fenbendazole-sulfone			10	500			100	500
Thiabendazole			100	100	50	100	100	100
Others								
Haloxon						100		
Levamisole				100		100		100
Morantel			50	800		700		

•MRLs in [µg/kg], adopted from (Kinsella *et al*., 2009; Durden and Wotske, 2009)

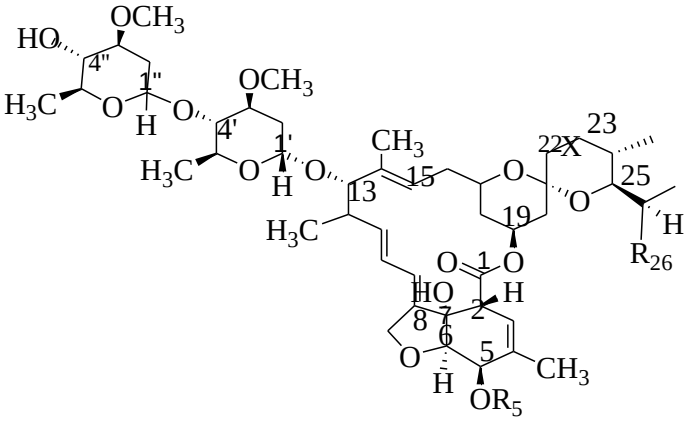
2.1.3 Ivermectin drug

Macrocyclic lactones or macrolides (MLs) are known for their strong anthelmintic properties. They comprise 2 groups: avermectins and milbemycin (Hennessy and Alvinerie, 2002). Their uses in veterinary medicine prevent internal and exterior parasite infection caused by larvae or adult types of parasites. They have for a long time demonstrated capacity to exterminate all exterior (ecto) and interior (endo-) parasites (Campbell, 2012; Campbell *et al*., 1984). Generally, MLs are a big complex ringed structure as illustrated by compound **1**. They are a 16-membered macrocyclic ring that has a disaccharide function, spiroketal group, and benzofuran ring (Campbell, 1989).



1

These macrolide endectocides are produced by a soil-dwelling bacterium, *Streptomyces avermitilis* as fermentation products (Campbell, 1989). This bacterium produces eight distinct avermectins, denoted by the letters A_{1a}, A_{1b}, A_{2a}, A_{2b}, B_{1a}, B_{1b}, $B_{2a,}$ and B_{2b}. A general structure for avermectin is depicted as compound **2** (Campbell, 2012)**.**



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Source: https://pubchem.ncbi.nlm.nih.gov/image/imgsrv.fcgi?cid=9832912andt=l

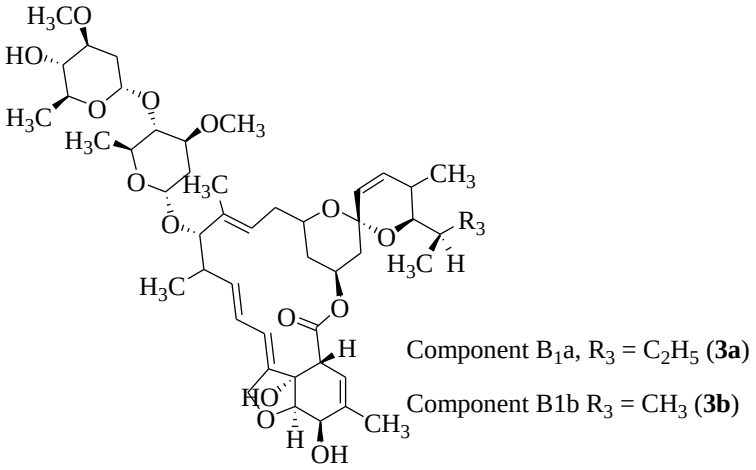
With reference to avermectin (**2**), the C5-position of the A-series molecules is methoxylated, while the B-series has the underivatized hydroxyl group. All molecules in the 1-subset have an olefinic link between C_{22} and C_{23}, whereas all compounds in the 2-subset have a "hydroxyl group" at position C_{23} due to the hydration of double bonds (Prichard *et al.*, 2012). The structural differences between the two subsets are depicted in table 2.3 with reference to compound 2 and compound 1.

Table 2.3: Structural differences between avermectin subsets 1 and 2-

Avermectin Subsets	R_2	R_{26}	C_{22}-X-C_{23}
A_{1a}	CH_3	C_2H_5	-CH=CH-
A_{1b}	CH_3	CH_3	-CH=CH-
B_{1a}	H	C_2H_5	-CH=CH-
B_{1b}	H	CH_3	-CH=CH-
A_{2a}	CH_3	C_2H_5	-CH_2-COH_2-
A_{2b}	CH_3	CH_3	-CH_2-COH_2-
B_{2a}	H	C_2H_5	-CH_2-COH_2-
B_{2b}	H	CH_3	-CH_2-COH_2-
Ivermectin	H	>80% C_2H_5 <20% CH_3	-CH_2-CH_2 -

The group of drugs falling under avermectins comprises; ivermectin-B_1a (**3a**), B_1b (**3b**) of compound **3**, abamectin (**4**), doramectin (**5**) emamectin (**6**), and eprinomectin B_1a (**7**), which contains two saccharide substituents at the C_{13} position. Besides, selamectin (**8**) falling under avermectins has one saccharide substituent (Campbell, 1992, 1989).

Moreover, Ivermectin, compound **3** is a semi-synthetic derivative of a naturally occurring macrocyclic lactone. Ivermectin is extensively used in veterinary medicine since it demonstrates a broad range of anthelmintic action. Additionally, it is used effectively for the treatment of human filarial worm infections. It is composed of two homologues that are distinguishable from one another by the presence of an alkyl group at position C26 (R_3). The ratio of the B_{1a} and the B_{1b} homologues is at least 80:20, respectively (Campbell, 1989).



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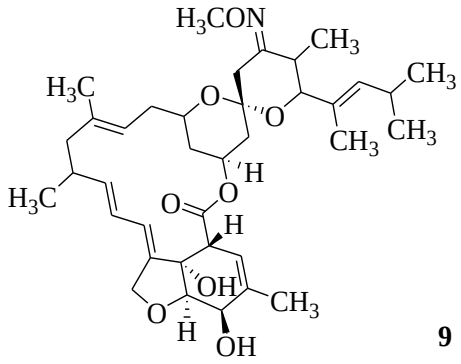
Source: https://www.sciencedirect.com/topics/medicine-and-dentistry/abamectin

The avermectin compounds contain a structure whose molecular masses and functional group in relation to compound **1** are shown in Table 2.4 (Durden and Wotske, 2009).

Table 2.4: Macrolide endectocides

Avermectin	**Molecular mass**	**Molecular formula**	**R_1**	**R_2**	**R_3**
Ivermectin B_{1a} (**3a**)	874.508	$C_{48}H_{74}O_{14}$	OH	H	CH_2CH_3
B_{1b} (**3b**)	861.079	$C_{47}H_{72}O_{14}$	OH	H	CH_3
Abamectin (**4**)	872.492208	$C_{48}H_{72}O_{14}$	OH	H	CH_2CH_3
Doramectin (**5**)	898.507858	$C_{50}H_{74}O_{14}$	OH	H	$-C_6H_5$.
Emamectin (**6**)	885.523842	$C_{49}H_{75}NO_{13}$	$-HN-CH_3$	H	$-CH_2CH_3$
Eprinomectin B_{1a} (**7**)	913.518757	$C_{50}H_{75}NO_{14}$	$-NHCOCH_3$		$-CH_2CH_3$
Selamectin (**8**)	769.440112	$C_{43}H_{68}NO_{11}$	OH	NH	$-C_6H_5$.

On the other hand, moxidectin, milbemycin, and nemadectin make up the milbemycin group. Moxidectin (**9**), a representative of the milbemycin that lacks the saccharide substituents (Molecular mass=639.377118; Molecular formula= $C_{37}H_{53}NO_8$)



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Source: https://pubchem.ncbi.nlm.nih.gov/compound/Moxidectin#section=2D-Structure

2.1.4 Synthesis of ivermectin

Ivermectin and abamectin, which were invented in the early 1980s, were the first macrocyclic lactones used in animal production. Abamectin was identified to have anti-nematode and anti-parasite properties in the Kitasato Institute's laboratories from a soil-dwelling bacterium, *Streptomyces avermitilis*, and then passed to Merck and Co., Inc.'s laboratories in 1974 (Stapley and Woodruff, 1982). Avermectins were found subsequently as a result of the deliberate selection of fermentation products for use in anthelmintic screening experiments (Campbell *et al.*, 1984). They were also made from the same bacterium due to its insecticidal and anthelmintic properties. Ivermectin influenced the production of animal parasite management, companion animal heartworm disease prevention, and human anti-filarial treatment (Campbell, 1989).

Avermectin B_{1a} (>90%) and B_{1b} (less than 10%). were found to be combined to form abamectin. Additionally, ivermectin was formed after selectively hydrogenating avermectin B1's *cis* 22, 23-double bond to produce 5-*O*-dimethyl-22,23-dihydro-avermectin B1a and B1b in an 80:20 ratio. As shown in figure2.1, this was accomplished using Wilkinson's catalyst, chlorotris (triphenylphosphine) rhodium (I)

[RhCl (PPh_3)$_3$], under conditions of H_2 pressures ranging from 1 to 150 bars and temperatures between 60 and 100 °C. (Campbell, 2012).

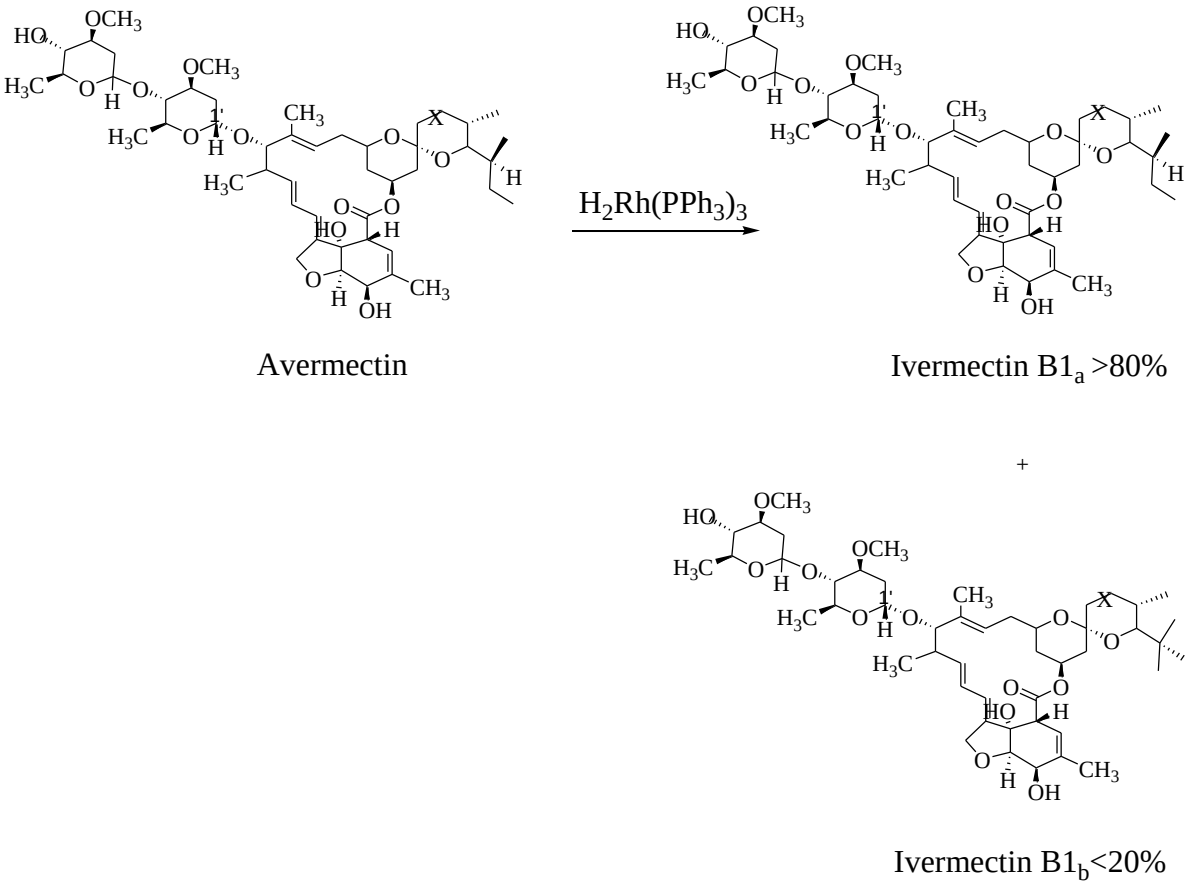


Figure 2. 1: Synthesis of ivermectin (Source: Campbell, 1989)

Ivermectin is composed of macrocyclic lactones having a combination of two components of homologous compounds. Combination of 22,23-dihydro avermectin B_{1a} (H_2B_{1a}, > 80%), and 22, 23-dihydro avermectin B_{1b} (H_2B_{1b}, < 20%) form the macrocyclic lactones that make up ivermectin. Components B_{1a} and B_{1b} have molecular formulas $C_{48}H_{74}O_{14}$ and $C_{47}H_{72}O_{14}$, and molecular masses of 875 and 860 respectively (Campbell, 1992). Chemically, ivermectin occurs as a combination of structural isomers that act in different ways and at different levels of toxicity. The first avermectin to be commercialized was the 22,23-dihydro-avermectin B_1 (Chabala *et al.*, 1980;

Egerton *et al*., 1979) and later in 1981, it was released for use in animals (Campbell, 1989).

All avermectins have high lipophilicity and thus can dissolve in a variety of organic chemical compounds, including alcohols, toluene, chloroform, methylene chloride as well as dimethylformamide. Their water solubility is quite poor. Additionally, they are sensitive to acid and exhibit moderate stability in a solution of anhydrous glacial acetic acid at room temperature. Aqueous acids such as HCl and H_2SO_4 as well as *p*-toluene sulphonic acid in methanol tended to cleave off the sugar moiety (Campbell, 1989).

2.2 Ivermectin residues in milk

Analysis to detect veterinary drugs residues present in foodstuffs and tissues of animal origin for consumption by humans, microbiological or bioassay screening procedures have been applied. However, some old methods failed to differentiate between members of veterinary drugs, in the classes of antibiotics. These methods gave a semi-quantitative estimate of the total of detected residues. The techniques provided semi-quantitative estimates of the number of detected residues (Stolker *et al*., 2008). Nonetheless, advanced and more sensitive methods are now available in the market that detect and quantify residues in food effectively and efficiently (Gennari *et al.*, 2016; Stolker *et al*., 2008; Lobato *et al*., 2006; Schenck and Lagman, 1999).

2.2.1 Depletion of ivermectin residues in treated animals

Veterinary medications have long been an important part of animal husbandry and/or modern food production, yet the drug's residues linger in foods derived from animals, posing a concern to food safety. Studies have indicated that most VDs administered to

an animal, after metabolization, leave residues in tissues. As a result, MRLs have been put in place in order to ensure consumer protection against the consumption of unsafe animal products. Similarly, food additive levels and veterinary medicine residual levels are determined using the same methods. However, the determination of veterinary drug residues and their metabolites is complicated by metabolic and dispositional processes that occur in the target animal (Kolberg *et al*., 2009).

Therefore, fundamental investigations on ivermectin excretion in milk and toxicological evaluation have become critical in this area of choice. Ivermectin gets excreted through mammary glands even after the legal withdrawal time (Toutain *et al*., 1988). Anastasio *et al.* 2002 reported that these residues remained in milk and plasma, as well as in mozzarella cheese made from buffalo milk, after administering subcutaneously, a single dosage of 0.2mg kg^{-1} on lactating animals.

Residue levels in milk have been observed to deplete gradually with maximum levels recorded as 2.8 ± 0.44 days after administration of the drug. Similarly, ivermectin has been detected in plasma on the 17.8 day and beyond 29 days post-treatment. This occurred after administering lactating cows with 0.2 mg/kg subcutaneously. Literature has it that 4.72 days is the mean milk depletion half-life. This means that a minimum of 47 days are required for the elimination of approximately 99% of the drug passing through the milk (Toutain *et al*., 1997). Furthermore, due to the nature of ivermectin, it is anticipated that about 5% of the administered medicine is secreted through milk (Canga *et al*., 2009; Toutain *et al*., 1988).

2.2.2 Presence of ivermectin in commercial cow milk

Milk has been found to contain ivermectin residues (Chicoine *et al*., 2007; Lobato *et al.*, 2006) and other farmed foods like salmon (Sanderson *et al*., 2007). Moreover, their presence and detection in food matrices is unacceptable, as the use of this drug in lactating animals meant to produce milk for human consumption, as well as usage on aquatic farming, is illegal (Davies *et al*., 1998). Regardless of the mode of administration, ivermectin residues turn toxic to other organisms like the dung-inhabiting insects (Martínez *et al*., 2017; Pérez-Cogollo *et al.*, 2015; Beynon *et al.*, 2012; Floate *et al.*, 2005; Lumaret *et al*., 1993).

Five macrocyclic-lactones that include; ivermectin, abamectin, doramectin, eprinomectin, and moxidectin were permitted for parasite management in cattle in Brazil. Besides ivermectin, which is the most widely used macrocyclic lactone in cattle, eprinomectin has been another medicine ratified for use on cattle providing milk for human consumption. Where all the lactones were used on lactating animals, milk had to be discarded since it was unfit for human consumption (Furlani *et al.*, 2015). Likewise, in the EU, ivermectin, doramectin, and abamectin in lactating animals have been prohibited due to their persistence. The use of eprinomectin and moxidectin was established on dairy animals at 20 mg kg^{-1} and 40 mg kg^{-1} respectively. The prescribed amount was reported to have safe levels of the drug residues (Danaher *et al.*, 2006). Unfortunately, eprinomectin production increased but turned to be expensive in comparison to other macrocyclic lactones. Consequently, most farmers opted to use cheaper drugs which led to the use of unapproved macrocyclic lactones whose consequences were dire (Nødtvedt *et al*., 2002; McPherson *et al*., 2001).

It has been reported that 5% of the injected drug of ivermectin gets eliminated through milk following monitoring of ivermectin in treated cattle (Imperiale *et al*., 2003). Literature has supported the observation by reporting contamination of milk in cases where sufficient time was not allowed to elapse between the treatment period and parturition (Chicoine *et al*., 2007). Monitoring of ivermectin milk residues in the Brazilian retail markets found that 17.8% of the tested samples were contaminated (Lobato *et al.*, 2006).

2.2.3 Effects of boiling and dilution of contaminated milk with residues

Research on contaminated cow milk with ivermectin residues has been carried out to examine the effects of boiling milk samples taken from treated cows (Avcı and Filazi, 2020; Imperiale *et al*., 2009; Cerkvenik *et al*., 2001; Rose *et al*., 1998). Besides pasteurization, cooking (boiling followed by cooling) has been a common method of preparing milk for consumption in most households. As a result, it would be necessary to interrogate the effects of industrial operations and domestic boiling on ivermectin residue stability. It is equally important to analyze the contents that consumers are exposed to on a qualitative or quantitative basis (Imperiale *et al*., 2009; Cerkvenik *et al*., 2001).

Past studies have shown that boiling reduces microbial contents and destroys whey proteins in milk and microwaving removes microbial populations but not whey proteins (Tremonte *et al.,* 2014). Cerkvenik *et al.* (2002) reported that heating sheep milk does not compromise the stability of ivermectin residues in the milk. Further, Imperiale *et al.* (2009) reported no chemical loss or bioconversion of ivermectin in raw sheep milk. This study will focus on effects of heating on ivermectin residues in cow milk.

2.3 Resistance of ticks to synthetic acaricides

Ticks are economically important among the ectoparasites because of their ability to spread diseases to both animals and humans. These external blood-sucking parasites infest mammals, birds, and reptiles thus transmitting protozoan, rickettsial, and viral livestock illnesses. These diseases are exceedingly expensive on a global scale (Rajput *et al*., 2006). According to Barker and Murrell (2004), there are around 867 different species of ticks, 683 of which are classified as hard ticks (Ixodidae) and the remaining 183 as soft ticks (Argasidae). The Argasidae family is divided into four genera; *Argas, Carios, Ornithodoros,* and *Otobius,* whereas the Ixodidae family has 241 species of Ixodes and 442 species from other genera. Among the most significant genera of hard ticks are *Amblyomma, Dermacentor, Haemaphysalis, Hyalomma, Ixodes, Rhipicephalus,* and *Boophilus* and approximately 10% of them serve as infection vectors for people and domestic animals (Jongejan and Uilenberg, 2004).

In Africa, global warming has played a significant influence in fostering the spread and colonization of new territories. Moreover, poor farm management, uncontrolled movements of livestock, and a profusion of wild animals interacting with livestock have contributed to the spread. These have pointed out to the most likely causes of the current complexity of control measures (Estrada-Peña and Salman, 2013). Ticks have been shown to be more common in subtropical and tropical climates despite the paucity of data, and they are present in about 80% of the cow population in the world (de Castro, 1997). Farmers have suffered significant financial losses as a result of heavy tick loads infesting livestock, resulting in blood loss, general stress from scratching and irritation, lowered productivity as a result of poor feeding, compromised immune function, damaged hides, and pathogen transmission (Valente *et al*., 2014; de Castro, 1997).

The *Hyalomma*, *Boophilus*, *Rhipicephalus*, and *Amblyoma* species can however transmit tick-borne *rickettsial* diseases like *Anaplasmosis* and *cowdriosis*, protozoan diseases like *Theileriosis* and *Babesiosis*, viral infections like Rift Valley fever, and tick-associated dermatophytosis. Tick infestation has been linked to health and management issues in many developing countries (Rajput *et al*., 2006; Jongejan and Uilenberg, 1994; Young *et al.*, 1988).

2.3.1 Rhipicephalus appendiculutus *(RA)*

The three-host tick *Rhipicephalus appendiculutus* (RA) (Plate 2.1), widely referred to as the brown ear tick, is a common species in Kenya.

Plate 2.1: *Rhipicephalus appendiculutus*- fed female (left), male (centre), and unfed female (right) (Photo by Mbugi, KU 2020)

RA causes significant economic losses by decreasing productivity, the cost of animals, and their skin quality. In Kenya, ticks are considered some of the most destructive and commercially significant livestock parasites (Mbogo, 1996). They prefer moderately cool, shaded, shrubby, or woody savannah or woodlands that experience a minimum of 24 inches of annual rainfall. They are prevalent in Eastern, Central, and South-Eastern Africa, especially at elevations above 7400 feet (2300 meters) above sea level, but the distribution is dependent on suitable environments and restricted by appropriate hosts (Walker, 2003).

As shown in figure 2.1, a three-host tick that detaches from an infected host at a stage of the RA life cycle spreads the cyclo-propagative, transcardially transmitted protozoan parasite known as *Theileria parva*. Besides, the deadly tick-borne disease (TBD) of cattle commonly known as East Coast fever (ECF) is transmitted by *Theileria parva* (Gachohi *et al.*, 2012; Jongejan and Uilenberg, 1994).

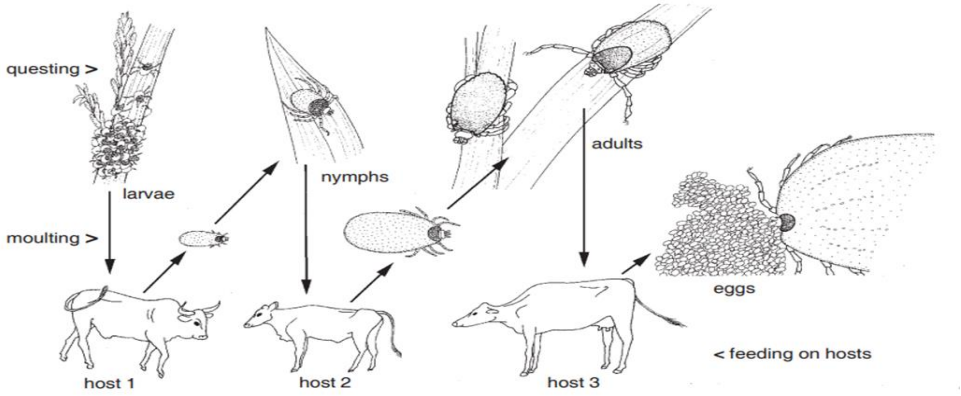


Figure 2. 2: The life cycle of *R. appendiculutus*

Except for ECF, all tick-borne infections are curable if arrested early enough. However, there is no effective treatment for advanced ECF cases. In most cases, infected cattle whose diagnosis has been delayed succumbs eventually (Gachohi *et al.*, 2012). Thus, careful planning is essential, including procedures taking into account other TBDs (Bishop *et al.*,2020). The tick remains attached to the host for several days while feeding before dropping to the ground to move on to subsequent life cycle stages. It infects a wide range of species, including cattle, buffalo, and huge antelopes. Besides, other livestock host species include sheep and goats. Moreover, immature ticks infest smaller hosts like hare and carnivores. Adult ticks prefer to attach themselves to the host's ears and feed there, while others feed on the host's head. In the subtropical regions of Africa, the completion of *R. appendiculutus* full life cycle is seasonal, thus occurrence of adults, nymphs, or larvae is compromised. A study observed that a tick

developed into an adult from mid-summer to late summer. Furthermore, in tropical places, one life cycle is completed each year, with up to three generations possible in locations with adequate rainfall (Walker *et al*., 2005).

2.3.2 Boophilus decoloratus *(BD)*

Boophilus decoloratus (Plate 2.2) is a known blue tick from its colour displayed by engorged females.

Plate 2.2: *Boophilus decoloratus* (Photo by Mbugi, Kenyatta University 2020)

B. decoloratus species are the most common one-host species of bovine ticks in Africa. Aside from cattle, they also infest and feed on goats, donkeys, sheep, horses, and wild ungulates. However, in comparison to other hosts, the tick prefers cow hosts. Thus, it searches for other hosts only when the preferred cattle species has been infested to the maximum. Moreover, different stages of this tick indicate preference on attaching and feeding on particular sites on the cattle. This tick attack occurs in the following order: back, upper legs, neck, shoulders, dewlap, and lastly the abdominal area (Walker, 2003). The one-host tick as indicated in figure 2.3 exhibits a monotropic type of behaviour where it completes lifecycle on one host. In about one week, engorged female ticks detach from the host. It lays approximately 1,000 to 2,500 eggs and depending on the environmental conditions, the eggs hatch in a record time of 3 to 6 weeks (Walker *et al*., 2005).

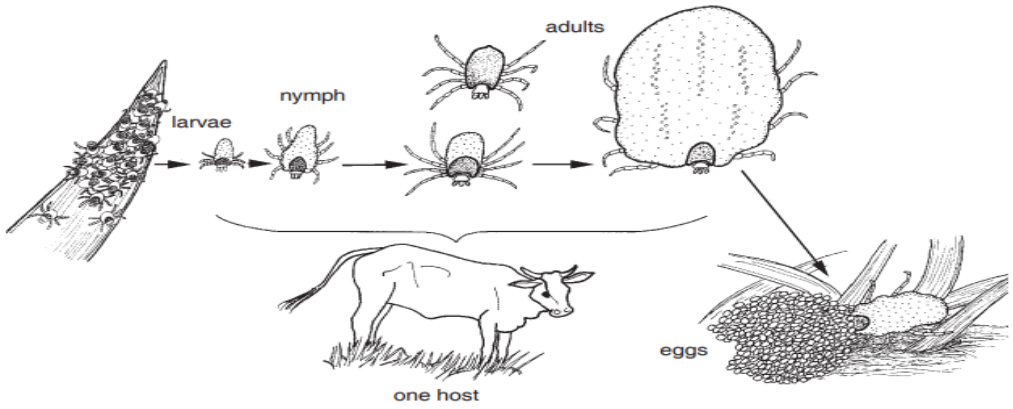
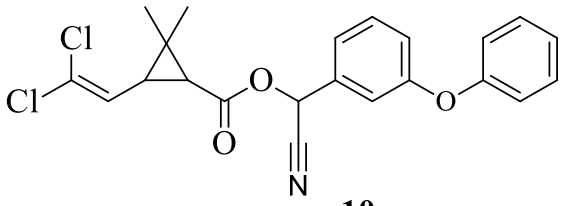


Figure 2.3: Life cycle of the one-host tick

As they wait for an ideal host, the larvae ascend and cling onto vegetation. The different stages of the tick spend approximately three weeks on the same host and moult to the succeeding stage until the life cycle ends. When fully fed and engorged, the female tick drops. The male tick for this species however remains on the hosts for a longer time. Apparently, this species has been reported to infiltrate savanna. Additionally, it is typically found in grassland and forested zones that are used as livestock pastures. Moreover, babesia transmission occurs throughout the nymph and adult phases, where ovarian transfer is from the previous generation. The species also transmit bacteria *Anaplasma marginale* which causes both *Bovine anaplasmosis* (gall sickness) and *Borrelia theileri* that leads to spirochaetosis in livestock (Walker *et al*., 2005; Walker, 2003).

2.3.3 Ticks control methods

The most common and efficient methods for controlling ticks and other flying insects include the use of chemicals, such as acaricides, but these methods have many drawbacks. According to studies, ticks are becoming resistant to a variety of acaricides. Some of these are; cypermethrin (**10**), chlorpyrifos (**11**) and amitraz (**12)** (Abbas *et al*., 2014; George *et al*., 2004; Martins *et al*., 1995).



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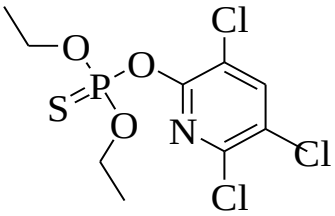

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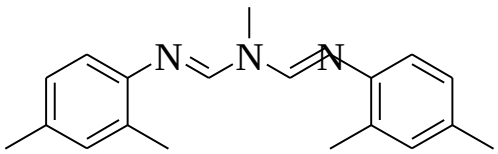

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The main downsides of their use are their toxicity and high cost. Their proclivity to accumulate in animal-derived foods along with the environment as residues is a matter of great concern. Consequently, there is a need to find other effective control techniques, such as vaccines (Rajput *et al*., 2006) or plant-based repellents safe for the ecosystem (Wanzala *et al*., 2018). Other research facilitated the use and development of efficient parasiticides, such as macrocyclic lactones, which changed animal husbandry and increased output (Danaher *et al*., 2006; Colebrook and Wall, 2004).

Table 2.5: Allowable MRLs of common acaricides

Active ingredient	**(µg/kg)**		**Codex**		
	Muscle	Liver	Milk	Fat	Kidney
Cypermethrin	50	50	100	1000	50
Deltamethrin	30	50	30	500	50
Cyhalothrin	20	20	30	400	20
Chlorpyrifos	10	10	10	10	10
Amitraz	50	200	10	200	200

Adopted from; Codex Alimentarius (2023).

2.2.4 Occurrence of tick resistance

Presently, various literature has documented numerous of instance of tick resistance to chemical control methods. The estimated prevalence of resistance varies in geography, the type of tick, and the acaricide used. A meta-analysis conducted by Dzemo *et al.* (2022) reported that the global resistance level of *R. (B.) microplus* was 66.2%. Further, 56.5%, 45.7%, 32.0%, and 27.4% of the included studies reported resistance against *R. (B.) decoloratus, R. appendiculatus, R. annulatus, R. evertsi evertsi, R. bursa, Hyalomma anatolicum, Amblyomma hebraeum,* and *A. cajennense* populations, respectively. In Benin, a study performed on 5 samples of *R. microplus* gathered from 5 farms in four to agro-ecological zones showed moderate to strong resistance to alpha-cypermethrin, deltamethrin, and amitraz (Adehan *et al*., 2016). Vudriko *et al.* conducted a study in Uganda that reported significant resistance among *R. appendiculatus* and *R. decoloratus.* Notably, 90.0% of the tick populations were resistant to synthetic pyrethroid, and 63.0% and 60.0% were 'super resistant' to deltamethrin and cypermethrin, respectively. Further, 43.3%, 13.3%, and 12.9% were resistant to synthetic pyrethroid co-formulations, chlorfenvinphos and amitraz, respectively. Worryingly, multi-acaricide resistance was identified in 55.2% of ticks (Vudriko *et al.*, 2016). In ranch in the semi-arid region of Paraíba State, Brazil, 96 %, 72 %, and 83 % of *R. microplus* ticks were reported to be resistant to cypermethrin, chlorpyriphos and amitraz (Vilela *et al.,* 2020), respectively.

Vilela *et al.,* (2020) conducted a study among in 26 ranches in the semi-arid region of Paraíba State, Brazil, that identified 92 % of *R. microplus* to ivermectin. In Punjab districts, India, larvae of all field collected *Rhipicephalus (Boophilus) microplus* were resistance to ivermectin (Singh *et al.,* 2015). El-Ashram et al. (2019) the first resistance

of ticks, *R. annulatus*, ivermectin in Egypt which occur among larvae hatched from field populations collected from Beni-Suef province. Further, *R. microplus* resistance to ivermectin has been reported in Mexico (Fernández-Salas *et al.*, 2012).

There is minimal literature regarding surveillance and the occurrence of tick resistance to chemical control in Kenya. The most recent study retrieved from literature is dated 1979 which reported that 99.3% of *Boophilus decoloratus* (Koch), 77.3% of *Rhipicephalus evertsi* Neum, and 62-5 % of *R. appendiculatus* Neum were resistant to toxaphene (Crampton and Gichanga, 1979). Mutavi *et al.* (2021) conducted a study in Laikipia, Central Kenya which identified critical misuse and indiscriminate application of acaricides in majorly amitraz- acaricidal regimens. This is a potential lead to selection for resistance (Githaka *et al.*, 2022). A farm-based longitudinal study in Western Kenya demonstrated that *Rhipicephalus* ticks developed resistance to amitraz overtime. Further, it was revealed that chlorfenvinphos was ineffective against amitraz-resistant *Rhipicephalus* tick populations (Kamidi and Kamidi, 2005).

It is vital to regularly examine the emergence of tick resistance to current acaricides to enhance antiparasitic control strategies (Abbas *et al.*, 2014; Molento *et al.*, 2013; Raynal *et al.*, 2013).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Experimental design

In order to detect and quantify ivermectin residues, qualitative and quantitative studies were performed. A questionnaire (annex 1) was developed and administered randomly to different farmers in selected farms in order to establish how they controlled ticks. Different farmers that employed ivermectin to treat animals subcutaneously were identified for sampling purposes. One farm was considered for monitoring from the day of treatment in order to collect samples and determine how residues dissipated in milk after animal treatment. Other samples were collected randomly from nearby farms and milk vendors from the market to assess if there was ivermectin contamination. Part of the samples collected were boiled and diluted to test the effects of boiling and dilution. Tick resistance to locally used synthetic acaricides (amitraz, cypermethrin, chlorpyrifos, and a combination of chlorpyrifos and cypermethrin) was assessed on *R. appendiculutus* and *B. decoloratus.*

3.2 Study location

The study selected different farms and milk selling points in Matura, Magumu ward, South Kinangop sub-county, Nyandarua County (figure 3.1), coordinates 0°49'43.4"S 36°35'13.3"E -0.828722, 36.587028. This is because Nyandarua County is a high- milk production zone in Kenya, with estimated annual amounts of approximately 40 million liters (Ministry of Livestock Development Department and Fisheries 2012). Sampling was done at Magumu area and the samples were collected between 27th June 2019 and 23rd August 2019. The samples were shipped to the Central Veterinary Laboratories in Kabete, where they were analyzed.

Republic of Kenya **Nyandarua County within the red demarcation**

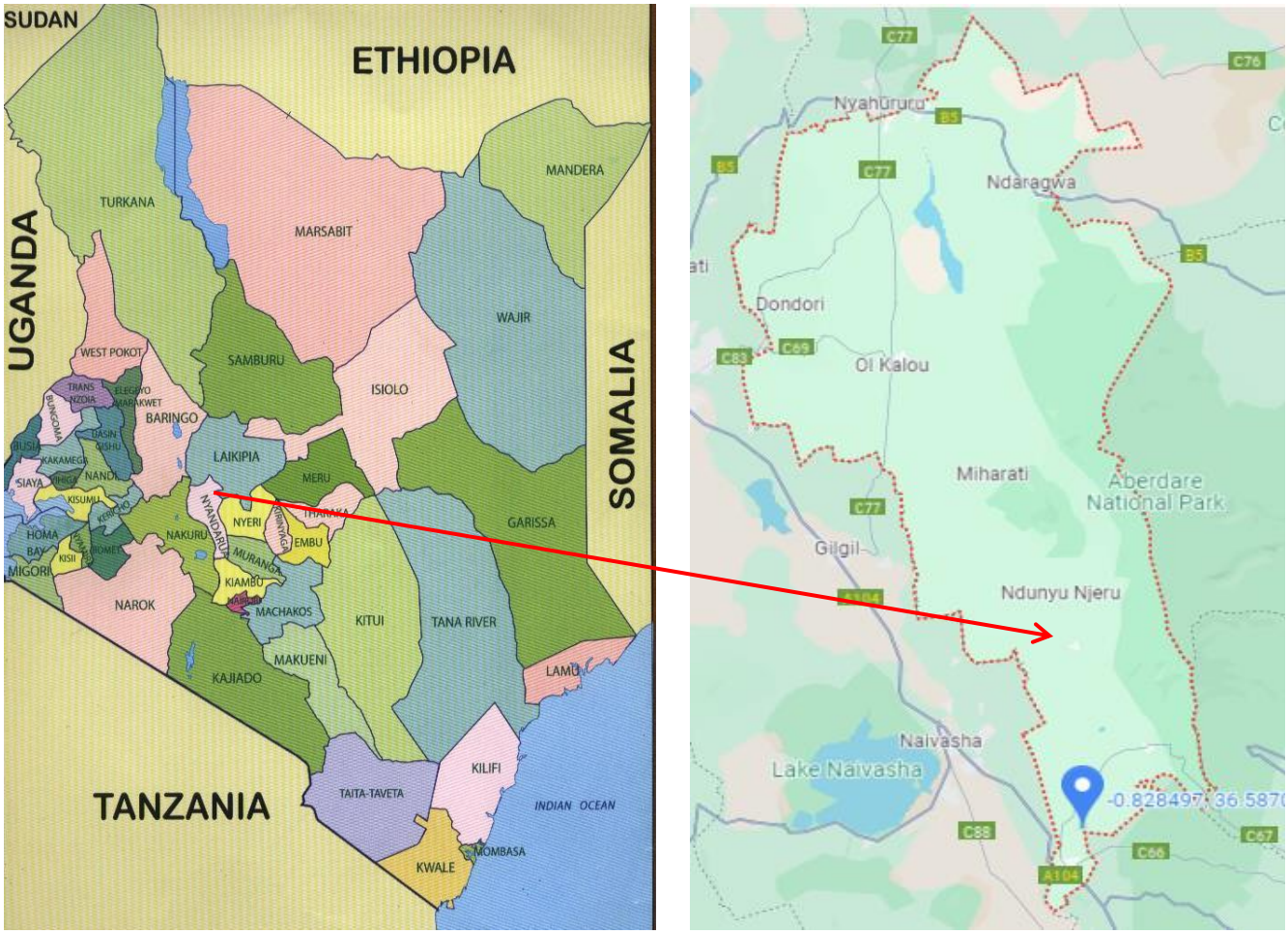


Figure 3.1: Map of Nyandarua County (https://maps-kenya-ke.com/map-of-kenya-counties)

3.3 Sampling

3.3.1 Milk samples

This study was guided by the responses obtained from the questionnaires for both purposeful and random sampling approaches. Blank samples were collected before treating a selected animal, three months' pre-treatment. The animal was then treated with 0.2 mg/kg of body weight of Ivermectin (1%). Five (5) ml of the drug was injected into the selected animal, which weighed 250kg. The samples were collected

consecutively for nine (9) weeks as follows; after 12 hours, 24 hours, and on days 3, 4, 6, 9, 12, 15, 17, 19, 21, 22, 26,29, 33, 36, 40, 43, 46, 47, 48, 50, 54 and 57 post-treatments. The sampling profile specified by Chicoine *et al*. (2007), Lanusse *et al.* (1997), and Toutain *et al*. (1988) was covered in the days. From the treated animal, 24 samples were collected. Following that, 125 samples were randomly collected from other farmers, local retailers, and pasteurized milk manufactured in the vicinity. The pasteurized milk was purchased in 250mL increments, whereas all other samples were collected in 50mL increments. Upon collection, these samples were shipped to the National Veterinary Reference Laboratories, Kabete at room temperature and refrigerated at -20℃ until use (Henshall, 2012; Cerkvenik *et al*., 2001; Monardes *et al*., 1996).

3.3.2 Tick samples

Female ticks were engorged at night and those that dropped off early in the morning from the selected animals were collected before treatment. Exactly 120 ticks were placed in a vented container with a layer of moist paper towel and some green grass. Then transported to the Acarology Laboratory, Central Veterinary Laboratories in Kabete. Here, their identity was authenticated with the help of Dr. Patrick Mbugi, an entomologist, Kenyatta University, following the guidelines provided by Walker (2003) on a guide for identification of tick species. They were kept away from chemicals, sunlight, and excess heat during transportation. To get rid of dirt, faeces, and other impurities, tick samples were thoroughly cleaned using distilled water. They were then gently dried with a paper towel before being sorted into distinct species and tested the following day (Drummond *et al.*, 1973).

3.4 Apparatus

Non-reusable Fluorinated Ethylene Propylene (FEP) centrifuge tubes (50mL) with screw caps, 2mL mini-centrifuge tubes, volumetric flasks (50ml, 100ml, 500ml, and 1000ml), spatulas, autosampler vials, micro-pipettes and disposable micro-pipette tips (1µL to 1000µL) (Sigma Aldrich, Germany). Syringe filters with 0.2 microns and 0.45 mm hole size, with a 25 mm diameter (Sigma Aldrich, Germany) were used in the study. Analytical weighing balance (Sartorius weighing balance Analytic AC 1205, Germany, model no. Analytic AC - 120 S), incubator (Memmert, Germany, model no. E511-0009), magnetic stirrer (Gerhardt Bonn, Germany, model no. 308786), magnets of various sizes (Sigma Aldrich, Germany), 50ml beakers or 100ml Pet Agro bottles (Sigma Aldrich, Germany), Petri dishes 150mm (Sigma Aldrich, Germany), Whatman Filter papers (Hardened ashless) Cat. No. 1541, 110mm circles (Sigma Aldrich, Germany), 5ml micropipettes, and 1ml micropipettes (Sigma Aldrich, Germany).

3.5 Chemicals and reagents

The chemicals and reagents that were used comprised Acetonitrile (MeCN) from Fisher Scientific, LC/deionized water from Merck KGaA, methanol (liquid chromatographic grade) sourced from Charlton Scientific Ltd, and 98% Formic acid (analytical reagent grade) from Sigma-Aldrich. Fisher Scientific provided pre-packaged quantities of 5g $NaSO_4$: NaCl (4:1 w/w). Pre-weighed mixes of 100 mg PSA sorbent (Sodium hydrogen citrate sesquihydrate 99 percent) with 300mg anhydrous sodium sulphate (Na_2SO_4), in 15 mL mini-centrifuge tubes. 99% Sodium hydrogen citrate sesquihydrate from Sigma-Aldrich. Ivermectin standard with a purity of 96.03 % was purchased from Norbrook Kenya Limited. Acaricides (amitraz, cypermethrin, and chlorpyrifos) were purchased from an Agro Distributor.

3.6 Instrumentation

The analysis was performed using an Agilent 1290 Infinity II liquid chromatography system (Agilent Technologies 1290 Infinity II liquid, Model no. 6490 Triple Quad LC/MS, California, USA) which consisted of an Agilent 6460 triple quadrupole mass spectrometer with Electrospray Interface (ESI), a binary pump, a vacuum degasser, a column oven, and an auto sampler. Additionally, acquisition and processing of data was done using the masshunter workstation program (Version B.08.00). The instrument has a sample management system with a 120-vial capacity. Agilent ZOBRAX Eclipse Plus C18, 5µm, 2.1 x 100mm column was used for separation and analysis. An analytical balance with a weighing capability of 180g and a precision of 0.01mg was used in the experiment, along with a vortex (Digisystem Laboratory Instruments, Model no. VM-2000-C, Taiwan), with a speed of 3,000 rpm. Further, extracts were centrifuged in a Heraeus multifyge X1R high-speed chilled centrifuge (Heraeus multifyge X1R high-speed, Model no. Thermos-scientific- 75004250, Germany) with a rotor head for eight 50mL sample tubes. Powersonic 405 benchtop sonicator (Powersonic 405 benchtop sonicator, Model no. Falc-Instruments-LBS1H10-0, Japan) was also used in this study.

3.7 Sample preparation

Residue chemists are progressively adopting LC-MS/MS techniques to detect veterinary drug residues both qualitatively and quantitatively (Kinsella *et al*., 2009; Stolker and Brinkman, 2005). For the confirmatory and quantification analysis of ivermectin residues in milk, this work optimized LC MS/MS - ESI techniques that were previously developed by Whelan *et al.* (2010), Dahiya *et al*. (2010) and Kinsella *et al*. (2009).

3.7.1 Experimental procedure for preparation of milk samples

Ten grams of milk samples were put into 50 mL centrifuge tubes. The analyte was then extracted by adding 10mL of acetonitrile (MeCN) and letting it stand for 10 minutes with the lid on. Thereafter, the mixture was gently shaken for a minute. Exactly 5g of Na_2SO_4: NaCl (4:1, w/w) was added and the resulting mix was once again mixed by shaking for 1 min (to prevent agglomerates from forming during the hydration of Na_2SO_4). Further, the mixture was partitioned by centrifuging at 4000 rpm for 5 min (Dahiya *et al.*, 2010; Kinsella *et al.*, 2009).

Afterward, 2mL of supernatant was added to a 15mL mini-centrifuge tube together with 50 mg of PSA sorbent (Sodium hydrogen citrate sesquihydrate 99%) and 150mg of anhydrous $NaSO_4$ for sample cleaning. To homogenize the sample extract, vortexing was done for a minute. Following that, the extract was put in a centrifuge and spun for five minutes at 4000 rpm. After transferring a 500µL supernatant aliquot into an autosampler vial and diluting it with 500µL of LC water, a 10µL aliquot was then injected into the LC-MS/MS system (Whelan *et al.*, 2010; Dahiya *et al.*, 2010; Kinsella *et al.*, 2009). The same extraction process was employed to test if boiling and dilution had any effect on the residues in positive samples with high residue levels (T2, T3, T4, T5, and T6).

3.7.2 Treatment of the tick samples

Drummond's adult immersion test method (Drummond *et al.*, 1973) was used to evaluate tick resistance to various acaricides. Using distilled water, 50 mL of four different acaricidal washes (combination, amitraz, chlorpyrifos and cypermethrin) were

made according to the manufacturer guidelines. Subsequently, each wash was vigorously stirred for 10 minutes with a glass rod to achieve solution homogeneity. The selected healthy and fully engorged female ticks were divided into four groups of ten ticks each and the weights of ticks in the set batches were recorded. Ticks from each batch were placed into a 50mL control solution and into the individual acaricidal washes (Singh *et al*., 2010; Jonsson *et al.*, 2000).

Ticks from various treatment groups were placed in the washes for 10 minutes, after which they were run through a filter that retained the ticks. Subsequently, they were dried on a clean paper towel and put on their backs in a Petri dish using double-sided adhesive (110mm diameter). For egg collection, a filter paper was placed beneath the ticks. For oviposition to occur, incubation was performed at controlled temperatures of 27 to 28°C and relative humidity levels ranging from 80 to 95 percent. Tick mortality was assessed on day 7 and all the adult ticks were discarded after 14 days. Eggs from each treatment batch were weighed, and the results were recorded. The eggs were put in an 8-drum shell vial with a perforated cotton fabric cover on top for hatching. On the 42nd day following incubation, the eggs were observed, and the percentage of hatched larvae was measured visually (Cutullé *et al*., 2013; Jonsson *et al*., 2000; Drummond *et al.*, 1973).

3.8 Preparation of mobile phases for analysis of ivermectin residues in milk

Two solutions, A and B, in a 5:95 ratio, were used as mobile phases in an isocratic flow. Solution A was made by dissolving 0.5 mL formic acid in 500 mL water (0.1 percent formic acid in water), while solution B was made by dissolving 0.5 mL formic acid in

500 mL methanol (0.1 percent formic acid in methanol). After that, the solutions were sonicated and refrigerated at 4°C for use during analysis (Dahiya *et al*., 2010).

3.9 Procedure for preparation of standard solutions

3.9.1 Preparation of ivermectin standard solutions

An amount of 0.960 mg of ivermectin standard was weighed, put in a 100 mL volumetric flask, dissolved in methanol, and diluted to volume. This provided a 100 µg/mL stock solution (100ppm), which was stored at 2° to 8 °C for subsequent use during analysis. A 1mL aliquot was measured from the stock solution diluted in 100 mL to the mark. This provided a 1 µg/mL (1 ppm) ivermectin standard solution concentration. Additionally, a series of calibration or standard solutions were prepared by taking appropriate aliquots of 1µg/mL standard solution. The aliquots were then diluted with methanol to produce a number of calibration standard solutions corresponding to 1, 2.5, 5, 10, 25, 50, 75, and 100 ng/m. The solutions were kept at a temperature of 2 to 8 °C (Kinsella *et al*., 2009).

3.9.2 Procedure for preparation of matrix-matched calibration standard solutions

In seven (7) separate centrifuge tubes, 10g portions of control or blank milk samples were weighed. The sample preparation procedures outlined by Whelan *et al*. (2010) and Kinsella *et al*. (2009) were then used to extract these samples. One (1) ml of the respective extract was transferred into the auto-sampler vials. Using the 1 µg/mL stock solution, appropriate volumes were pipetted to make a total volume of 1ml of matrix-matched calibration levels of 1, 2.5, 5, 10, 25, 50, 75, and 100 ng/mL. The 1 ml aliquots were diluted using LC water. Hence, a matrix-matched calibration curve was developed

using matrix-matched calibration standards to demonstrate how the matrix affected the target analyte.

3.9.3 Procedure for preparation of solvent calibration standard solutions

Appropriate aliquots were obtained from the 1µg/mL ivermectin standard stock solution. Using methanol, the aliquots were further diluted to volume to prepare standard calibration solutions with concentrations of 1, 2.5, 5, 10, 25, 50, 75 and 100 ng/mL. The solutions were kept at a temperature of 2 to 8 ℃ (Cheng and Liu, 2014; Whelan *et al.*, 2010; Dahiya *et al.*, 2010; Kinsella *et al.*, 2009).

3.10 Procedure for preparation of spiked samples

Twelve (12) blank samples of milk weighing 10g each were placed in 50 mL centrifuge tubes. The formula used to calculate the volume of the spiking solution is the amount of the spiking solution (volume) = {(desired spike concentration x volume of the sample)/Concentration of the spiking solution}. The appropriate calculated amount of 10 ng/ml of ivermectin standard solution was added to each sample. The mixture was then left to stand for 15 minutes. The analytes were extracted, then an aliquot of 500 µL was diluted with 500 µL of LC water, thereafter 10 µL was injected into the LC-MS/MS instrument (Whelan *et al.*, 2010; Dahiya *et al.*, 2010; Kinsella *et al.*, 2009).

3.11 Liquid chromatography parameters

Agilent ZOBRAX Eclipse Plus C18, 5µm, and 2.1 x 100mm column was employed in this study for chromatographic separation. In addition, mobile phases that consisted of two solutions A and B as stated in section 3.8, were run in an isocratic mode, in the ratio 5:95 respectively. The temperature of the LC column was fixed at 40℃. By

introducing 10.00µL of 10µg/mL into the ESI interface, optimal parameters were reached.

3.12 Mass spectrometry (MS) parameters

The MS experiments used air pressure and the positive electrospray ionization (ESI+) mode, with nitrogen gas serving as the collision gas, as well as for nebulization and desolvation. The results in form of a mass spectra for MS were generated in a scan mode that ranged from m/z 100 Da to 20,000 Da. Similarly, mass spectra acquired for protonated molecules of ivermectin were recorded using a product ion scan method in the masses that ranged from 100 Da to 20,000 Da. The work identified three fragments in Multiple Reaction Monitoring (MRM) mode within a 50 milliseconds dwell time. Two characteristic qualifier fragments, 897.5>329 and 897.5>240, and 1 quantifier 897.5>183, were monitored for ivermectin. Given that the most abundant ion obtained was 897.5>183, it was picked as the quantifier ion. Following method optimization, collision energy of 68V and fragmentor voltage of 150 Kv were recorded. Further, the following parameters were optimized and applied in the study: Capillary voltage (V) of 4000, Gas Flow of 8 l/min, Gas Tempof 325 ℃, Nebulizer 35 psi, Sheath Gas Heater 325°C and Sheath Gas Flow of 12 l/min.

3.13 Method optimization for liquid chromatography-tandem mass spectrometry

3.13.1 Qualitative analysis

A volume of 50µL of 10µg/ml ivermectin standard was loaded into the LC-MS/MS at a dead volume. To determine the most abundant molecule or precursor ion and the fragmentor voltage, a scan was performed in the positive Electron Spray Ionisation (ESI+) mode with masses tuned to a range of between 50 to 2000. 10µl of 1µg/ml of

the solvent standard was re-injected. The scan was done at MRM mode to get the retention time. Precursor ion was fragmented further to obtain products ions. Both fragmentor voltage and the collision energies were varied until the precursor ion was fragmented into product ion(s). The matrix effect was evaluated by comparing the two calibration curves generated i.e. matrix-matched and solvent calibration curves. This guaranteed that this study was free from interferences brought on by the occurrence of other matrix components (Dahiya *et al*., 2013; Kinsella *et al*., 2009).

3.13.2 Quantitative analysis

Whelan *et al.* (2010), Dahiya *et al.* (2010), and Kinsella *et al.* (2009) techniques were used for quantitative analysis, with minor adjustments. Na_2SO_4 was used instead of $MgSO_4$. Furthermore, the samples were doubly diluted upon extraction, and the samples were analyzed using 0.1% formic acid in water as solvent A and 0.1% formic acid in methanol as solvent B in a 5:95 ratio as mobile phases. The adjustments to the approach were validated using the 2002/657/EC guidelines where a number of parameters were determined. The characteristics examined were linearity, selectivity, and specificity, as well as recovery, accuracy, repeatability, and analytical limits (limit of quantitation).

3.13.2.1 Linearity and matrix effect

By preparing eight-point matrix-matched and solvent calibration curves at different concentration levels, the linearity was assessed. Starting with 1ng/ml, concentrations were increased to 2.5, 5, 10, 25, 50, 75, and 100ng/ml. A "linear least-squares regression analysis" was used to examine the recorded data for linearity for each level of the calibration standard after they had been run in triplicate.

The linear regression line was defined as "*y= ax + b*," where "*y*" was the detector response, "*x*" represented the concentration of an analyte, "*a*" was the slope, and "*b*" represented the y-intercept of the best line of fit. In reference to Chhonker *et al*. (2018a), Zhou *et al*. (2017), Dahiya *et al*. (2013) and Kinsella *et al*. (2009), the matrix effect was evaluated by comparing the two produced curves. However, sample preparation and clean-up steps outlined in the procedure for Quick, simple, cheap, effective, robust, and safe (QuEChERS) were suggested to reduce matrix effects by reducing or eliminating interfering matrices (Kinsella *et al*., 2009; Zhou *et al*., 2017). Additionally, the extracted materials were diluted with methanol in the ratio of 1:1, before injection, to reduce the matrix effect (Stahnke *et al*., 2012).

3.13.2.2 Selectivity and specificity

Selectivity was determined through analyzing eight (8) blank samples of milk that were spiked with ivermectin standard. This was done to evaluate the effects of potentially endogenous substance interferences. The study applied a calibration curve that consisted of eight non-zero concentrations (1, 2.5, 5, 10, 25, 50, 75, and 100ppb). Consistent with the acceptance criteria for the correlation coefficient (r^2) for standard calibration curves (Chhonker *et al*., 2018a; Kinsella *et al*., 2009), the study recorded and employed linearity for the analyte of 0.998, which was nearly a perfect linearity of 1. Further, using the previously described procedure, the chromatograms from both blank and spiked samples were compared. Besides, by injecting the standard and looking for interference peaks throughout the prescribed retention period, the specificity of the assay was evaluated for the existence of chromatographic

interferences that could be present in milk samples (Dahiya *et al*., 2013; González and Herrador, 2007).

3.13.2.3 Precision

Two metrics, repeatability and within-laboratory reproducibility, were employed to assess the degree of precision. Twelve (12) samples of milk spiked with 10ng/ml of ivermectin standard were tested. The acquired data was expressed as relative standard deviations (RSD). The percentage RSD value for retention time, concentrations obtained, and recoveries were determined. A precision limit of ≤20 % of RSD was required to meet acceptance criteria (Chhonker *et al*., 2018a; Dahiya *et al.,* 2013; Kinsella *et al*., 2009). Further, as an additional way of determining precision, the study applied the Horwitz equation as follows: "RSD (%) =2^ (1−0.5logC) where C = concentration of the analyte in the sample as a decimal fraction" (Horwitz, 1982).

3.13.2.4 Recovery

The efficiency and effectiveness of the extraction procedure used in the veterinary medicine under evaluation was assessed using twelve (12) blank milk samples spiked with 10 ppb of the standard. Equation 3.1 was used to calculate the recoveries of ivermectin in spiked samples (Kinsella *et al.,* 2009).

$$\text{Recovery (\%)} = \frac{\text{Analytical Result}}{\text{True Value}} \text{ x } 100 \qquad \text{Equation 3.1}$$

3.13.2.5 Estimation of Limit of Detection (LOD) and Limit of Quantification (LOQ)

To calculate LOD, the research applied a signal-to-noise (S/N) 3:1 ratio for the strongest mass transition relative to background noise from the blank. In addition, LOQ

was determined using a 6:1 signal-to-noise (S/N) ratio (González and Herrador, 2007). In a different way, LOD was determined using the equation given; LOD= ksy/x/m, where k=3, $s_{y/x}$ is the residual standard deviation of the regression line, and m is the calibration graph's slope (Miller and Miller, 1993).

3.14 Data validation

3.14.1 Validation of data for LC-MS/MS technique

Analytical grade standards were employed to calibrate the analytical instruments that were used in this study to establish the various parameters necessary for analysis. The LC-MS/MS was calibrated by initializing the instrument. This was followed by optimization to get the method parameters, product ion, precursor ion, fragmentor voltage, collision energy, and retention time. Optimization was achieved by injecting 50 µl of 10 µg/ml of the standard ivermectin on a dead volume. Masses were scanned to find the precursor ion with the highest abundance. The column was returned and 10µl of 1µg/ml of the solvent standard was injected. The scan was performed at MRM mode to attain retention time. Collision energies along with fragmentor voltages were further varied to attain the most abundant fragments (product ions). Instrumental errors were minimized by checking on instrumental drifts through the running of a blank sample after every 5 samples. The tests were all performed in triplicate. The quantification of the results was determined by the use of calibration curves whose linearity was tested by coefficient regression. To test the hypothesis, single-and two-factor analysis of variance (ANOVA) was performed, along with the comparison of means and standard deviation. Quantitative data was expressed as mean±SEM

3.14.2 Bioassay procedure of the tick resistance

The efficacy of test acaricides was determined through a comparative study using a procedure adopted from Drummond *et al.* (1973), Singh *et al*. (2010) and Jonsson *et al.* (2000). Each batch of treated ticks had their estimated reproduction (ER) compared to the control. ER was a calculation of how many larvae each female produced at the acaricide concentration utilized in the bioassay. The ER was estimated as indicated in equation 3.2.

$$\text{ER} = \frac{\text{weight of eggs (g)x Estimated hatch x 20,000 (\# eggs per g)}}{\text{No.of female ticks}} \qquad \text{Equation 3.2}$$

The ER of each treated tick group was then compared to the computed ER for the control group. Equation 3.3 illustrates how to calculate the control percentage:

$$\text{Control (\%)} = \frac{\text{ER for control ticks} - \text{ER for treated ticks}}{\text{ER for control ticks}} \text{ x } 100 \qquad \text{Equation 3.3}$$

Where resistance (%) = 100 - Control (%)

For interpretation of data: 50-80% indicated emerging resistance

While > 80% indicated resistance

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Introduction

The study used both qualitative and quantitative evaluations to identify and quantify ivermectin residue analytes in milk.

4.2 LC-MS/MS method performance optimization

4.2.1 LC-MS/MS qualitative analysis of ivermectin residues in milk.

The study detected only a limited mass-to-charge ratio range and obtained the most abundant precursor ion m/z 897.5 at a voltage of 150V and at an acquisition time of 4.754 mins. This is as indicated in the mass spectra shown in figure 4.1 and in the chromatogram appearing in figure 4.2.

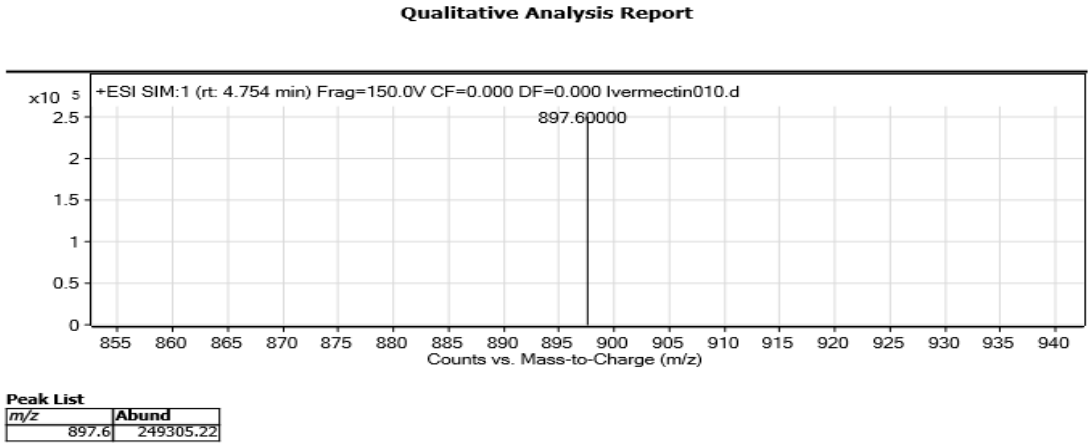


Figure 4. 1: SIM mass spectrum of m/z 897.5 precursor ion

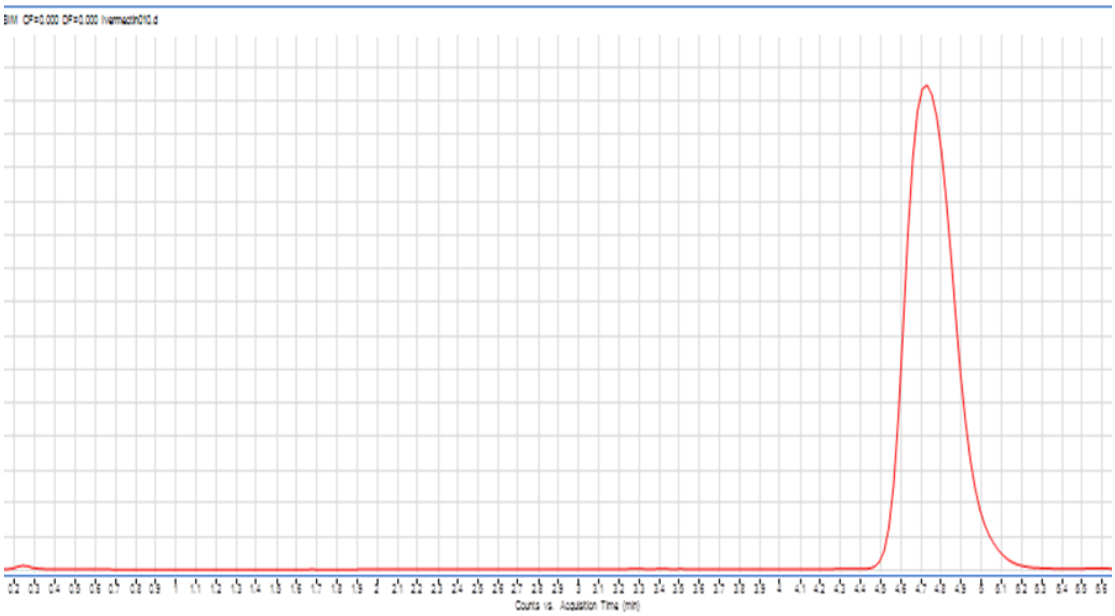


Figure 4. 2: SIM chromatogram of m/z 897.5 precursor ion

Further, the system was run in MRM mode, and as shown in Figure 4.3, the precursor ion reported in SIM mode was likewise identified at m/z= 897.5, albeit with a longer retention time (approximately 8 minutes) than in SIM mode.

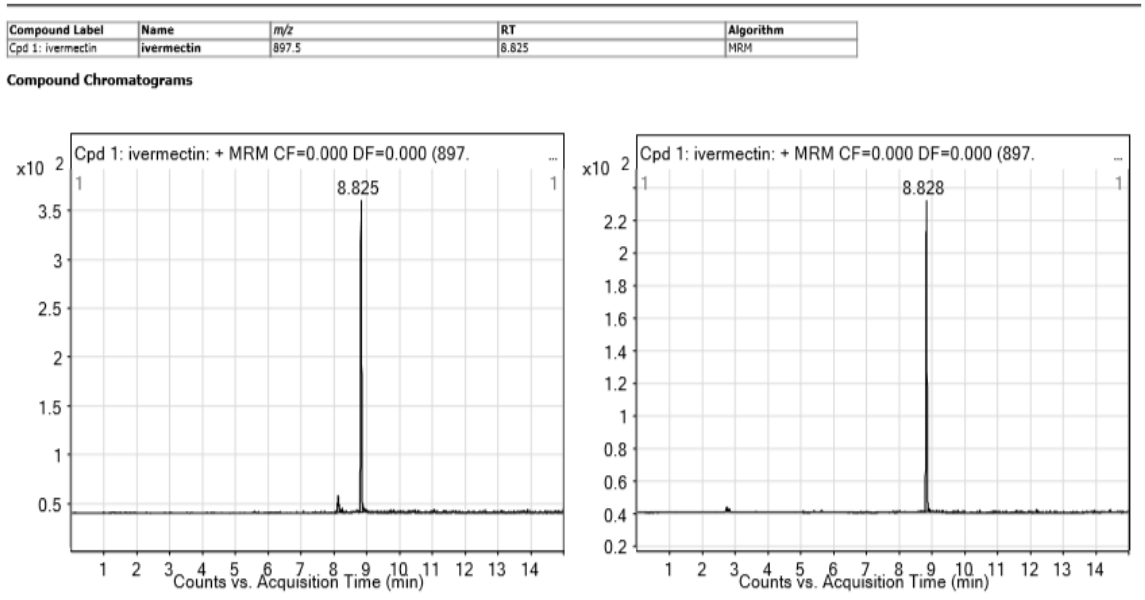
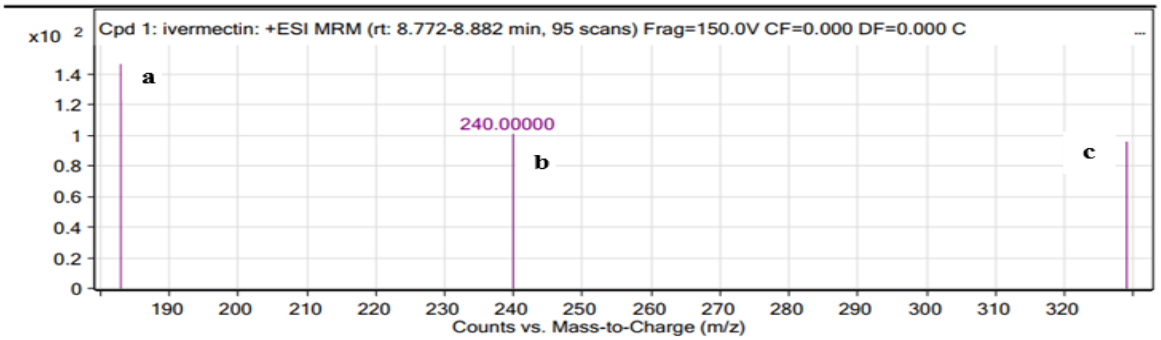


Figure 4. 3: Retention time for m/z 897.5 precursor ion in MRM mode

Since ivermectin's molecular weight was 875 g/mol, the study alluded that the base peak for the primary mass spectrum m/z= 897.5 ($M + Na^+$), resulted from the chelation of ivermectin molecule with a sodium ion (Na^+). This is because, ivermectin is composed of two chemically modified avermectins with molecular weights of 875 and 860: at least 80% of 22, 23-dihydro-avermectin-B1a (H2B1a) and >20% of 22, 23-dihydro-avermectin-B1b (H2B1b) respectively (Campbell, 1992; Fisher and Mrozik, 1992). According to studies, the H2B1b molecule metabolized faster than the H2B1a homolog (Gonzalez-Canga *et al.,* 2009). Because of this, the primary metabolite detected *in vivo,* H2B1a, which was the most prevalent residue found in body fluids, accounted for a significant portion of the dose that was eliminated in unchanged form (Lobato *et al.*, 2006; Markus and Sherma, 1992).

When the precursor ion (m/z 897.5) underwent further fragmentation, this research recorded the elution of three product ion transitions at a retention time below 9 minutes, and at 150V. Consequently, for qualitative analysis and chromatographic peak identification, the retention time ranged from 8.782 to 8.858 minutes, with a precision of 0.08. Annexure 3 indicates sampled chromatograms for acquisition/ retention time obtained for ivermectin. Figure 4.4 displays the peaks for the three product ions within the acquired retention time.

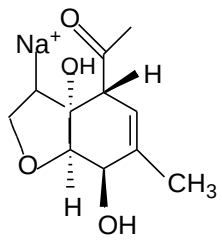


Peak List

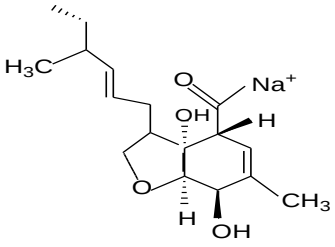
m/z	Abund
183	146.04
240	100.96
329	95.44

Figure 4. 4: Mass spectrum of ivermectin fragments/product ions

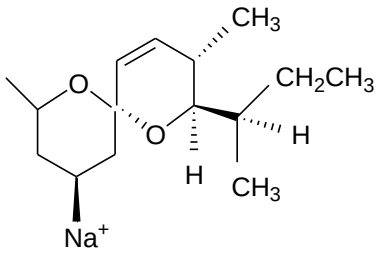
This enabled the identification and confirmation of ivermectin residues in milk. These product ion transitions comprised of ivermectin fragments as illustrated in **13** (897.5 > 183), **14** (897.5 >240), and **15** (897.5 >329).



13



14



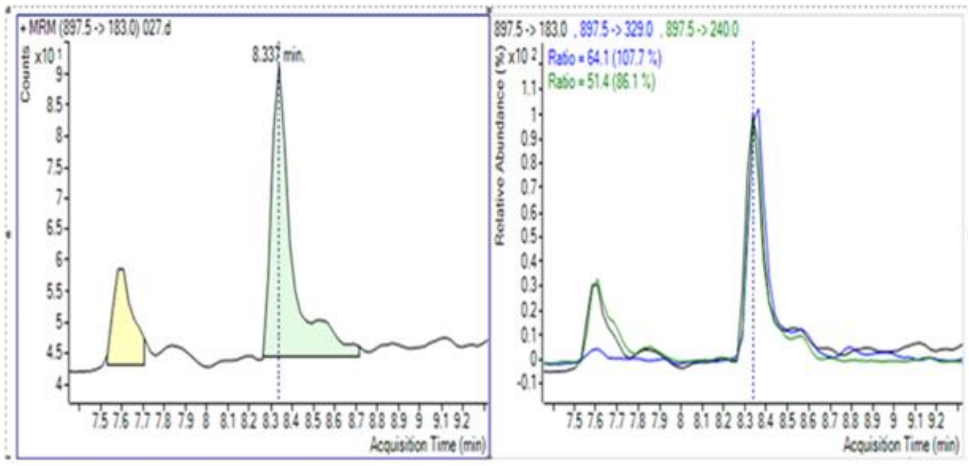
15

As shown in Figure 4.4, the product ion **13** with a transition of m/z183, was the most abundant with a relative abundance of 146.04. This ion was used as a quantifier for quantitative analysis. Whereas, product ions **14** and **15** with transitions of m/z 240 and m/z 329, and abundances of 101.96 and 95.44, respectively, were used as qualifier ions for qualitative analysis. Similarly, the chromatograms for the product ions reported at m/z values of 183, 240, and 329 are presented in annexure 4.

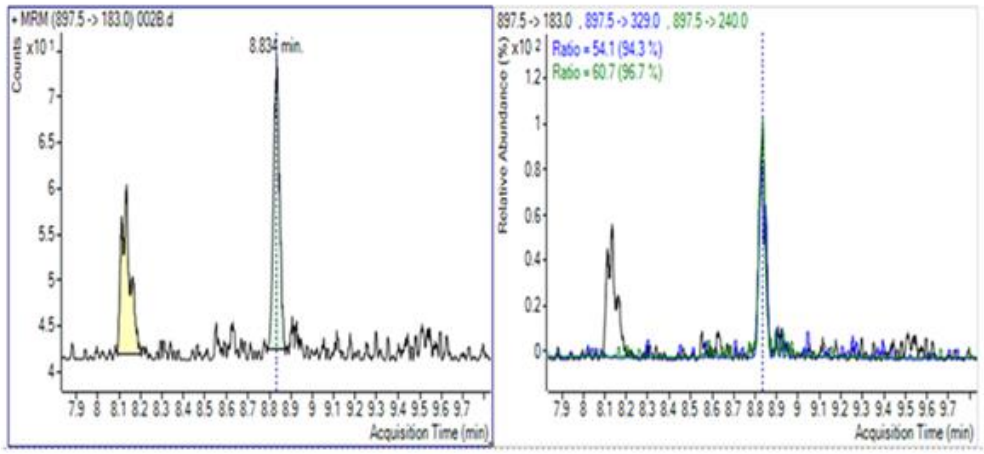
The most abundant secondary mass spectrum from m/z 897.5, with a base peak at m/z 183 which can be seen in Figure 4.4 as well as in chromatograms in annexure 4, was determined to represent the benzofuran moiety present in ivermectin molecules that broke at C-1 and C-9 and chelated with Na^+ [160 + Na^+]. The different ends of the moiety caused the bonds to break at these sites in the ivermectin molecule rather than on the disaccharide moiety. This is because the disaccharide has two ends: a non-reducing end that can be used to substitute *O*-atom on the anomeric carbon and a reducing end that is linked to the rest of the ivermectin molecule. At the reducing end,

the *O*-atom on the anomeric carbon was un-substitutable (Calvano *et al*., 2017). In addition, the product ion **14**, m/z 240 was identified as the benzofuran moiety that broke from the main ivermectin molecule at C-1 and at C-13, which bonded the disaccharide moiety. Similarly, it chelated with the sodium ion [217 + Na^+]. Besides, the parent ion, m/z 897.5, gave rise to another unique product ion with a base peak at m/z 329 (**15).** This product ion was the spiroketal moiety of the ivermectin molecule chelating with the sodium ion [306 + Na^+]. As a result, the spiroketal moiety broke from the main structure at C-19 that attached to oxygen at C-1 and C-16. Consequently, the study adopted the m/z= 329 and m/z= 240 product ions as qualifier ions, while m/z183, which was the most abundant, was selected as the quantifier ion.

In addition, the mobile phases were evaluated for efficiency in ionization and separation of the analyte. 5mM ammonium formate mobile phase was used as stated in literature (Kinsella *et al.*, 2009). However, poor separation of peaks was observed for the 5ppb ivermectin standard used, as seen in the peaks posted in Annex 5. The performance of a different mobile phase, notably 0.1% formic acid in water and 0.1% formic acid in methanol was also evaluated in the study. At a retention time of fewer than nine minutes, well-resolved peaks in the positive ionization were produced in an isocratic flow of 5: 95. This was tested on a concentration of 1ppb solvent and matrix-matched ivermectin standards. The chromatograms for 1ppb solvent and matrix-matched standards obtained were shown to be well resolved as illustrated by figures 4.5 **a** and **b**, respectively.



a)



b)

Figure 4. 5 a, b: 1ppb chromatograms in solvent and matrix, respectively

The study further tested the same solvent system at a lower concentration of 5ppb of matrix-matched standard. Equally, the peaks were successfully integrated as presented in Annex 6. This indicated that there was no suppression or enhancement of signal in the matrix-matched curve and so the method's analytical performance remained unaffected. As a result, this mobile phase solvent system was optimized since it achieved efficient ionization and separation of the analyte.

4.2.2 Quantitative analysis

4.2.2.1 Linearity and matrix effect

The research showed that the responses for different concentrations of; 1.0 ng/mL, 2.5 ng/mL, 5.0 ng/mL, 10 ng/mL, 25 ng/mL, 50 ng/mL, and 100 ng/mL were; 0, 1.607, 4.2427, 9.482, 23.692, 50.600, 64.903, 85.896 in matrix-matched calibration and 0, 2.612, 5.520, 6.771, 21.561, 51.702, 65.939 and 103.734 in solvent calibration signals. In order to determine whether the data were linear, linear least-squares regression analysis was used. A line was fitted to the data using the equation $y = ax + b$, and the values a and b that represented the slope and y-intercept of the line, respectively, were calculated. The values for a and b obtained from the solvent calibration data were 1.049 and 1.579, respectively, while the values obtained via matrix matching for a and b were 0.874 and 0.707, respectively. The matrix-matched calibration curve seen in Figure 4.6 was used in this study for the quantification, whereas the solvent calibration curve is posted in Annexure 7.

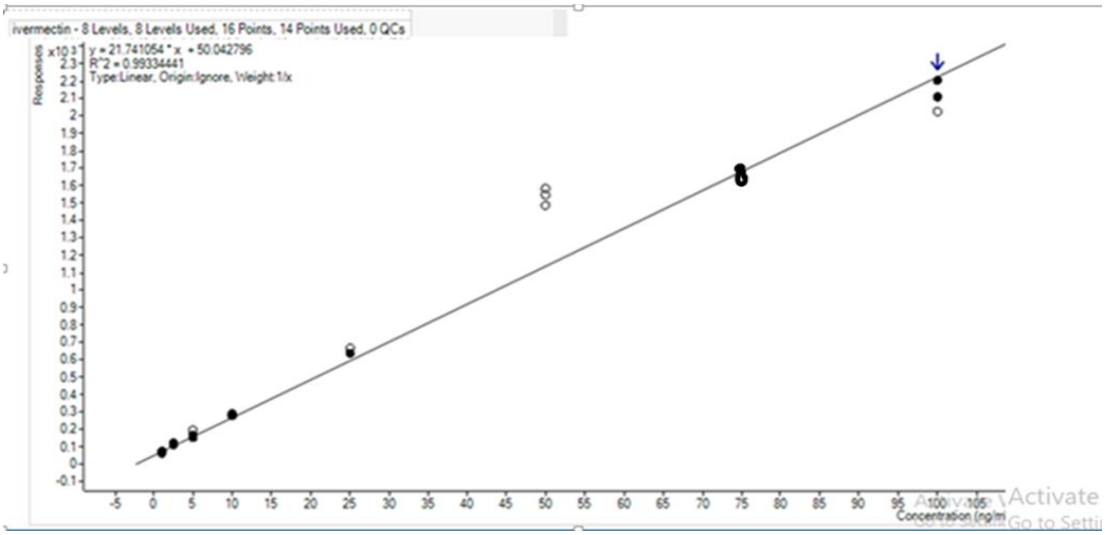


Figure 4. 6: Matrix-matched calibration curve

It was demonstrated that both of the calibration curves made using ivermectin standard solutions were linear in the concentration ranges of 1ng/mL to 100ng/mL. Further,

solvent and matrix-matched calibration curves had correlation values of 0.998 and 0.993, respectively. The QuEChERS pesticide extraction method, which is effective, was used in the analysis. The method was considered to have effective sample preparation and clean-up processes that was regarded to be strategic for removing co-eluting components. The extraction method was recommended during chromatographic analysis (Anastassiades *et al.*, 2003).

Before injecting a volume of 10 µL, the extracted samples were mixed in a 1:1 ratio with LC-MS grade water. A wash solvent was also administered to dissolve any residual sample in the LC system after each sample was injected. Blank milk samples were run after fifteen (15) samples to check contamination; however, there were no quantifiable amounts of the residue found. This is because quantitative studies using LC-MS/MS(ESI) have found the matrix effect to be a serious flaw. Since the matrix effect was considered to either increase response (response/ion enhancement) or decrease reaction (ion suppression), consequently, it is necessary to consider matrix effects when validating an LC-MS method (Zhou *et al*., 2017). Although they cannot be eliminated, they can be reduced by enhancing sample preparative processes in addition to optimizing LC and MS settings. In addition, if an analyte's concentration is excessively high, the injected volume is reduced and/or the samples are diluted. These are some simple approaches for lowering the amount of co-eluting components and, as a result, lowering matrix effects (Lien *et al*., 2009). The findings of the study showed that there was no matrix effect, meaning that the matrix had no effect on the instrument's response, either enhancing it or suppressing it. Hence, quantitative work employed the matrix-matched calibration curve.

4.2.2.2 Selectivity and specificity

To illustrate the method's selectivity, blank milk samples (n =8) spiked with amounts of the ivermectin standard solutions were examined. The calibration curve obtained indicated a correlation coefficient of r^2 = 0.993 and it was then determined that they were free of endogenous substances that caused contamination. Kinsella *et al*. (2009) stated, “Due to the high selectivity of the MS in MRM mode, peaks that eluted close together could be clearly distinguished based on their different precursor and product ions”.

Chromatographic interference peaks at the established experimental retention time were examined by comparing two chromatograms produced using a 10 ppb solvent and a 10 ppb matrix-matched standard. The observed chromatograms are presented in Figures 4.7 and 4.8.

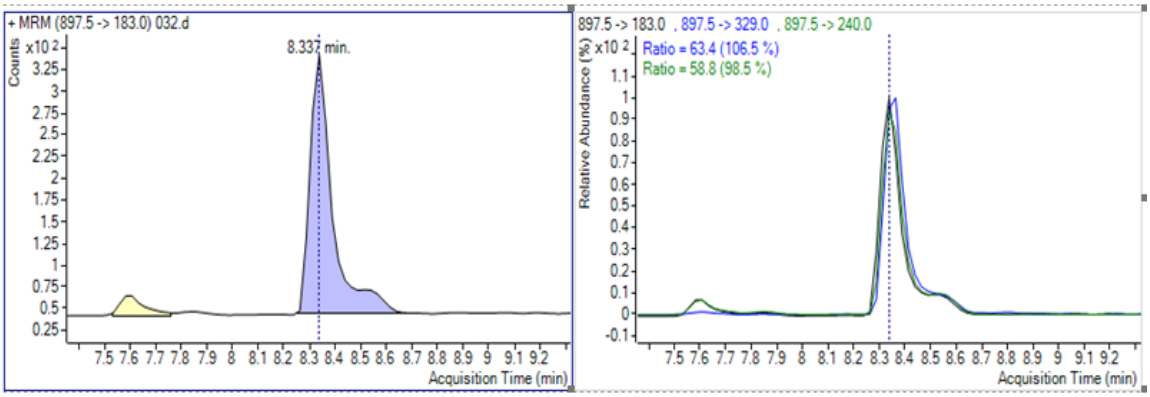


Figure 4. 7: 10ppb solvent chromatogram

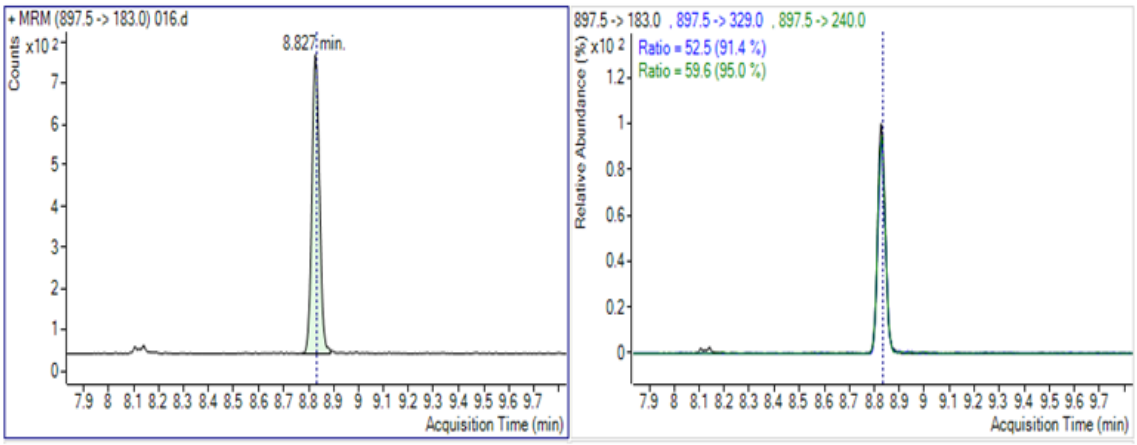


Figure 4. 8: 10ppb matrix-matched chromatogram

The analysis also examined for specificity, and it found no competing peaks that co-eluted with the experimentally measured analytes. As a result, the analyte's quantitative results for ivermectin determination were unaffected.

4.2.2.3 Estimation of LOD and LOQ

The lowest measurable concentration was identified to be 10 ng/ml and was validated using standard solutions at 2.5 ng/ml. The LOD for the strongest mass transitions was reported to be 2.5 ng/ml. Similarly, the results were confirmed using a matrix-matched 10 ng/ml reference solution. The equation: $LOD = ks_{y/x}/ m$, was used to compute the LOD. This resulted in a value of 2.664, which was then rounded to the nearest concentration level used, 2.5 ng/ml.

Additionally, since the recovery rates obtained from the study ranged from 74.997 to 101.435 % as earlier discussed, with CV less than 20%, the spike concentration can be applied as the LOQ, qualifying the 10 ng/ml level. This is in line with the recommendations in the document SANTE/12682/2019, EURL | Pesticide Residues | Analytical Quality Control and Method Validation Procedures for Pesticide Residues Analysis in Food and Feed (EURL).

4.2.2.4 Precision

The capacity of the procedure to produce identical findings for preparation of the sample was measured by repeatability. Twelve replicate sample determinations with results shown in Table 4.1 were considered for the process. From the values obtained in Table 4.1, the study obtained method repeatability for the concentrations at 2.810%. The precisions achieved for the analysis in % Relative Standard Deviation (RSD) of average values for retention times, concentrations, and recoveries were ,0.08, 10.419 and 10.419 respectively (table 4.2).

Table 4. 1: Values for retention times, concentrations, and recoveries

Samples (10ppb)	RT	Conc.	% recovery
Spike 1	8.362	9.412	94.12
Spike 2	8.365	8.613	86.131
Spike 3	8.363	8.528	85.267
Spike 4	8.363	9.306	93.06
Spike 5	8.362	9.014	90.136
Spike 6	8.363	10.112	101.12
Spike 7	8.365	10.144	101.435
Spike 8	8.367	7.794	77.939
Spike 9	8.363	8.988	89.884
Spike 10	8.366	7.498	74.997
Spike 11	8.363	7.783	77.828
Spike 12	8.341	10.101	101.01
Average	**8.362**	**8.941**	**89.409**

Table 4. 2: Precision for retention times, concentrations and recoveries

Variable	Mean ± SEM	CV (% RSD)	SD	Sr
Retention time	8.362±0.002	0.08	4.59E-05	0.018
Concentrations	8.941±0.269	10.419	0.868	2.416
% Recovery	89.410±2.69	10.419	86.768	24.155
Theoretical RSD	3			
% Repeatability	2.810			
LOQ	Set at 10 ppb			
Matrix effect	-19.957 (no matrix effect)			

All the parameters had a precision of < 20%. The values were within the acceptable tolerances of < 20% as indicated in the European Commission's 2018 document's criterion for precision acceptance. Furthermore, the Horwitz equation was used to compute the allowable maximum repeatability, and the maximum RSD for the data was found to be 3%. At a 95% level of confidence, the computed repeatability standard deviation (SD) for concentration was 2.416. According to Horwitz (1982), RSD was acceptable because it was less than the theoretical value of 3%. Thus, the acceptability of the results was also based on the Horwitz equation.

4.2.2.5 Recovery

The recoveries for ivermectin in spiked samples were calculated to measure extraction efficiency as well as the analyte that reached the end of the procedure in the determination of ivermectin. These concentrations were determined by spiking 12 blank samples with 10 ppb of the standard. In milk samples, the percentage recoveries of ivermectin ranged from 74.997 to 101.435 %. On average, concentration recovery was shown to be 89.409% (Table 4.1 section 4.2.2.4). Recovery rates of less than 100% and greater than 100% were attributable to within-run precision/repeatability, which includes contributions from any aspect of the technique applied that varied within a run. This may include effects from gravimetric and volumetric inaccuracies, heterogeneity of the test material, and variance in the chemical treatment steps during analysis which are mostly reflected in the dispersion of replicated results (Thompson *et al*., 2002).

Nevertheless, signal suppression and enhancement effects was witnessed in biological matrices (Becker *et al*., 2004). LC-MS was also shown to suffer from matrix effects, particularly when using ESI to analyze complex matrices extracts, despite being a

sensitive and selective analytical technique (Matuszewski *et al*., 2003, 2003; Kebarle and Tang, 1993). However, the results met the 70-120% "analytical quality control and method validation processes for pesticide residues analysis in food and feed" criteria, which have been suggested for implementation by January 1, 2020. In addition, the precision for recoveries was obtained at 10.419 % RSD (table 4.2). This was below the required level of 20%.

4.3 Ivermectin residue levels in milk samples obtained from farms and the market

Ivermectin was detected in measurable concentration levels in 55.84 % (n = 77) of samples collected from farms. The quantities ranged from a minimum of 0.05±0.01 ng/ml to a maximum residue concentration of 96.02±2.42 ng/ml. In contrast, 29.87% (n=48) of the market samples had a maximum average concentration of 97.76±5.49 ng/ml and a lowest value of 3.22±0.59 ng/ml (table 4.4). Besides, ivermectin residual levels in some farm milk samples were found to decrease with time. However, this was not observed in the positive samples that were tested from the market. As a result, it was evident that farmers in the area were certainly administering the drug to their dairy cows. Among the 48 samples collected from the market, 21 samples were from retail shops (SC and SD) that sold processed milk from a local dairy processor. Similarly, they recorded ivermectin residue levels that ranged from 3.22±0.59 ng/ml to 97.76±5.49 ng/ml. This signaled a violation in the use of the drug where no MRLs are allowed in milk by Codex, the EU, and the US tolerance guidelines. They all recommend that no amount of this drug should appear in milk.

Table 4.3: Ivermectin residue levels in milk samples from farmers and markets

	Farm samples			**Market samples**	
Sample ID	Mean Concentration (µg/ml)	Sample ID	Mean Concentration (µg/ml)	Sample ID	Mean Concentration (µg/ml)
FA/1	42.22±2.10	FD/3	44.37±2.37	SB/4	39.53±0.45
FA/2	0.44±0.12	FD/4	45.25±3.96	SB/5	7.34±0.32
FA/4	4.21±0.30	FD/5	42.67±3.36	SC/2	7.58±0.16
FA/5	0.52±0.19	FD-6	59.60±0.64	SC/4	31.15±1.72
FA/6	0.06±0.01	FD/7	57.01±1.29	SC/6	53.30±5.10
FA/7	0.28±0.10	FD/8	4.06±3.60	SC/7	97.76±5.49
FA/8	1.74±0.12	FD/9	37.24±1.41	SC/8	8.32±1.54
FA/10	0.05±0.01	FD/10	49.37±0.36	SD/3	10.01±0.87
FA/11	1.90±0.51	FD/11	42.34±1.31	SD/4	27.24±3.34
FA/13	0.54±0.33	FD/12	42.04±1.60	SC/10	3.22±0.59
FA/15	0.77±0.56	FD/13	50.50±0.78	SC/12	8.91±0.57
FA/16	46.33±1.08	FD/14	49.70±1.61	SC/13	3.72±2.55
FB/3	26.71±0.36	FD/15	57.52±2.82	SC/15	74.72±8.73
FB/4	50.17±4.68	FE/1	0.11±0.00	SD/5	4.80±1.52
FB/5	39.44±1.88	FF/1	0.58±0.17		
FB/6	34.97±1.34	FF/2	2.98±0.188		
FB/7	41.20±1.86	FF/3	5.41±0.58		
FB/8	40.44±0.56	FF/4	10.85±0.24		
FC/11	41.96±3.65	FF/5	27.67±0.43		
FC/12	37.57±0.27	FF/6	69.26±1.25		
FD/1	15.71±2.56	FF/7	96.02±2.42		
FD/2	22.80±1.1				
Total number of farm samples tested					**77**
Number of contaminated farm samples					**43**
% contaminated farm samples					**55.84%**
Total number of market samples tested					**48**
Number of contaminated market samples					**14**
% contaminated market samples					**29.87%**

According to Toutain *et al*. (1988), the breast gland produced 5.5% of a subcutaneous dosage. In light of the high lipophilic nature of ivermectin drug, which resulted in the existence of ivermectin residues together with extended persistence in milk, farmers are encouraged to refrain from using the drug on nursing animals (Canga *et al*., 2009). Other research findings have reported presence of macrocyclic lactones, leading to a ban from human consumption (Cheibub *et al*. 2019; Macedo *et al*. 2015; Furlani *et al*. 2015; Cerkvenik *et al*. 2004; Imperiale *et al*. 2004). Similarly, countries for instance

the USA do not allow detectable levels of ivermectin residues occurring in milk (Whelan *et al.*, 2010; Kinsella *et al.*, 2009).

4.4 Time-dependent depletion of ivermectin residue excreted through milk from treated animals

Milk samples were analyzed using the suggested method for extraction to detect if ivermectin was excreted through milk and how it dissipated with time. Milk samples were monitored for 57 days following SC treatment of dairy cattle. The concentration of tests performed 12 hours after treatment revealed amounts of the residue at 24.09±0.48 ng/ml, whereas the highest mean concentrations of 60.90±0.98ng/ml were recorded on day 2, shown in figure 4.9.

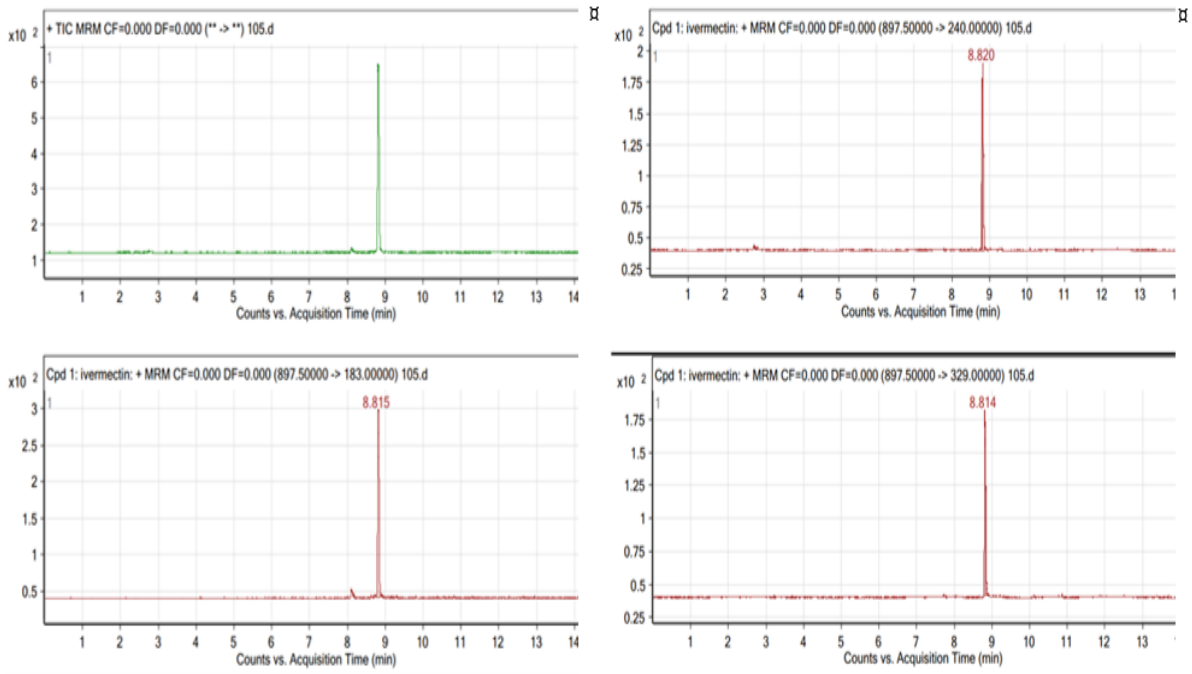


Figure 4. 9: Ivermectin residues chromatogram for day 2 post-treatment

Moreover, the lowest detectable concentrations of 0.95ng/ml were obtained on day 17 after treatment. The chromatograms in figure 4.10 show the concentration on day 17 in detail.

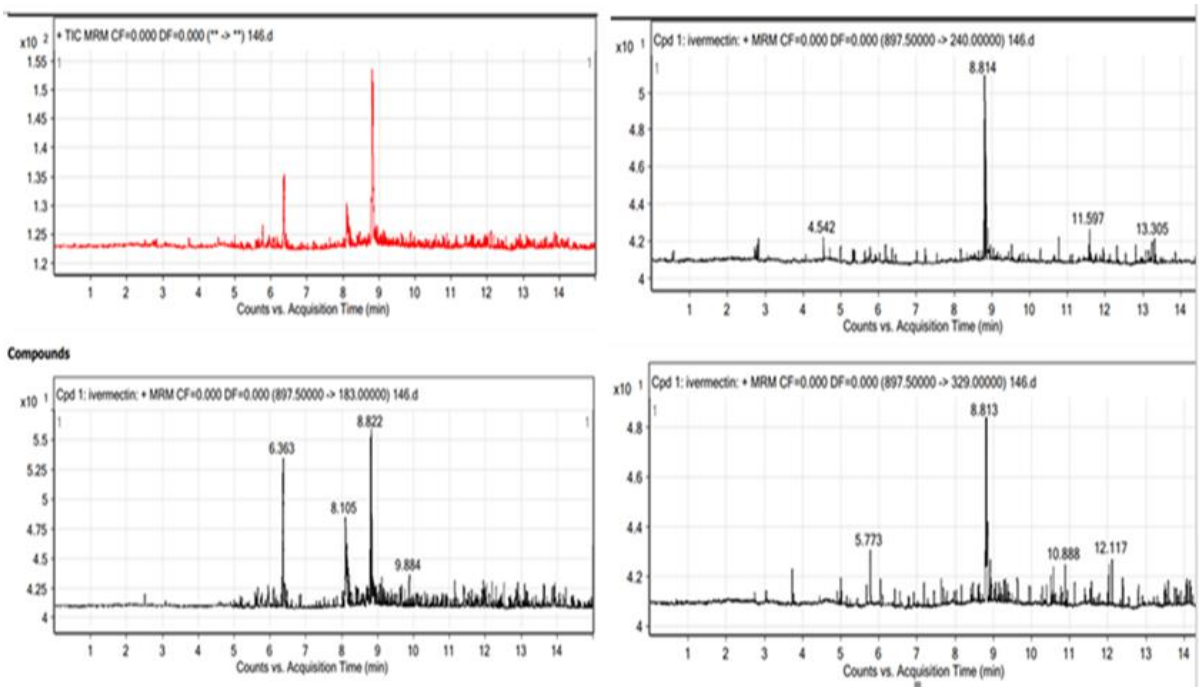


Figure 4.10: Ivermectin residues chromatogram for day 17 posttreatment

The study established that ivermectin residues were released from mammary glands through milk. The residue was excreted in milk from day 0.5 to the 17th day following treatment in quantifiable amounts. Although the signal response was weak at low concentrations (as seen on day 17), this was attributable to noise and endogenous chemicals, and the peaks that eluted close together were recognized by their distinct precursor and product ions (Kinsella *et al*. 2009). However, all the ions were distinctly observed and the optimized retention time remained unchanged.

From these results, ivermectin residues were noticeably eliminated through milk, thus the study further examined the data obtained, which is presented in Table 4.3. This was done in order to determine the pattern of ivermectin residue depletion and the time at which it occurred in cow milk. The obtained pattern suggested that residues were present in raw milk samples for 17 days (0.95±0.11ng/ml), after which no residues were

detected. On day two after treatment, the highest mean values of 60.90±0.98 ng/ml were found, with a subsequent trend toward decreasing levels.

Table 4.4: Ivermectin mean concentrations in milk from treated dairy cows

Day (after treatment)	Concentration of Ivermectin (µg/ml)
0.5	24.09 ± 0.48^{d}
1	43.39 ± 1.39^{c}
2	60.90 ± 0.98^{a}
4	49.18 ± 1.57^{b}
6	24.42 ± 1.11^{d}
7	23.95 ± 1.29^{d}
9	19.37 ± 1.63^{d}
12	4.23 ± 0.33^{e}
15	1.47 ± 0.53^{e}
17	0.95 ± 0.11^{e}
18	$0.\ 00\pm0.\ 00^{e}$

Means followed by different small superscript letters column-wise denotes significant difference ($p<0.05$)

Similarly, Escribano *et al.* (2012) and Anastasio *et al*. (2002) reported equivalent trends and levels of ivermectin in animal tissue, particularly milk. However, on the contrary to this study, Alvinerie *et al.* (1994) reported the highest amounts of milk on the third day following subcutaneous treatment with ivermectin (Alvinerie *et al.*, 1994). The null hypothesis was therefore rejected since some means on some days were significantly different as denoted by different small superscript letters. The observed trend in the study was also represented graphically as shown in Annex 8.

It is worth noting that the study identified ivermectin residues in milk samples collected from dairy cows after the prescribed amount of 200 µg/kg was administered subcutaneously. This matched the findings recorded in literature (Canga *et al*., 2009; Anastasio *et al.*, 2002; Toutain *et al.*, 1997, 1988; Alvinerie *et al.*, 1994). Ivermectin, a large and lipophilic molecule is relatively insoluble in water. However, due to its pharmacokinetic behaviour, lipophilicity suggests a distinctive distribution and thus,

slow elimination of the molecule excreted without metabolization (Cerkvenik *et al*., 2004; Dahiya *et al*., 2010).

Nonetheless, other studies have shown that ivermectin remains in dairy animal milk after SC treatment (Canga *et al.*, 2009; Chicoine *et al*., 2007; Lobato *et al.*, 2006; Anastasio *et al*., 2002; Alvinerie *et al*., 1994). It is important to note that, a drug's formulation and administration methods have an influence on its pharmacokinetics. According to other related studies, subcutaneous administration provided the highest bioavailability, followed by oral administration. This is because; ivermectin persisted in the body for a long time due to its slow plasma clearance and ability to accumulate in adipose tissue. Importantly, ivermectin accumulated in subcutaneous tissues due to its weak water solubility (Gonzalez-Canga *et al*., 2009), allowing for delayed absorption from the injection site. As a result, the drug is able to stay in the bloodstream for a long period of time (Gonzalez-Canga *et al*., 2009). Likewise, when examining ivermectin excretion-time profile from cattle dung, Fernandez *et al.* (2009) observed a similar profile/result which was in a different matrix.

4.5 Ivermectin residue levels after boiling and diluting milk samples

Comparative analysis of raw, boiled, and diluted milk samples (T2, T3, T4, T5, and T6) revealed detectable quantities of ivermectin residue. Similarly, Ivermectin residues were found in different quantities in spiked blank milk samples. The levels of concentrations for the three replicates of each sample and the computed averages are shown in Table 4.5.

Table 4. 5: Concentrations (ng/ml) for T2-T6 raw, boiled and diluted milk samples

Sample		Raw		Boiled		1st Dilution		2nd Dilution	
	Replicate	Conc.	X̄	Conc.	X̄	Conc	X̄	Conc	X̄
T2	Rep 1	41.39		40.60		29.61		17.62	
	Rep 2	42.72		38.35		26.81		13.69	
	Rep 3	46.06	43.39	41.12	40.03	28.17	28.2	14.54	15.28
T3	Rep 1	56.74		60.74		35.45		10.8	
	Rep 2	72.69		70.31		36.77		10.68	
	Rep 3	68.72	66.05	65.31	65.45	36.82	36.35	10.8	10.76
T4	Rep 1	59.9		57.00		25.05		3.97	
	Rep 2	62.86		58.97		22.9		2.55	
	Rep 3	59.94	60.9	57.58	57.85	23.9	23.95	3.17	3.23
T5	Rep 1	23.25		20.83		2.7		0	
	Rep 2	23.38		23.50		2.31		0	
	Rep 3	26.64	24.42	24.07	22.80	2.81	2.61	0	0
T6	Rep 1	26.51		21.97		0.32		0	
	Rep 2	22.85		20.90		0		0	
	Rep 3	22.48	23.95	23.00	21.95	0	0.11	0	0
Blank	Rep 1	0		0		0		0	
	Rep 2	0		0		0		0	
	Rep 3	0		0		0		0	0
Spiked	Rep 1	9.49		8.1		4.26		1.67	
(10ppb)	Rep 2	10		7.9		3.97		2.05	
	Rep 3	9.35	9.612	8	8.001	4.53	4.25	0.60	1.44

To evaluate performance of the analysis, blank and spiked milk were tested alongside test samples. The five test samples (T2, T3, T4, T5, and T6) were also examined for the effects of boiling on ivermectin residues present. In spiked blank milk samples, the signals were observed in raw and in all the other three treatments. Table 4.6 and Annex 9 summarize the results collected.

Table 4. 6: Ivermectin levels after boiling and diluting milk samples

Time (Days)	Raw	Boiled	1st Dilution	2nd Dilution
T2	43.39 ± 1.39^{bA}	40.03 ± 1.60^{cA}	28.20 ± 0.81^{bB}	15.28 ± 1.19^{aC}
T3	66.05 ± 4.79^{aA}	65.45 ± 2.76^{aA}	36.35 ± 0.45^{aB}	10.76 ± 0.04^{bC}
T4	60.90 ± 0.98^{aA}	57.85 ± 0.70^{bA}	23.95 ± 0.62^{cB}	3.23 ± 0.41^{cC}
T5	24.42 ± 1.11^{cA}	22.80 ± 0.61^{dA}	2.61 ± 0.15^{dB}	BDL
T6	23.95 ± 1.29^{cA}	21.95 ± 0.56^{dA}	0.11 ± 0.01^{eB}	BDL
Spiked (10ppb)	9.612 ± 0.32^{A}	8.001 ± 0.13^{A}	4.25 ± 0.21^{B}	1.44 ± 0.36^{C}
Blank	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

*BDL (Below Detectable Limits) Means followed by different small and capital letters in a column and row respectively indicate statistically significant at $p<0.05$ level

Based upon the quantities of ivermectin residues reported for spiked samples, data from the comparative analysis demonstrate no significant difference in raw and cooked milk samples. Similarly, there was no significant difference in the concentrations obtained for treated raw samples to those of corresponding boiled samples. As a result, it was determined that almost the entire residue was effectively recovered and that there was no denaturing or chemical loss of ivermectin metabolites during the boiling process. Moreover, there were no metabolic products of analyte degradation detected in the chromatograms obtained from boiled, blank and in spiked milk samples analyzed. Therefore, the study ruled out the possibility of any potential endogenous influence. This alluded that ivermectin was thermally stable to the primary boiling process practised on milk in most households. The results also demonstrated that uncooked milk may be used to assess customers' overall exposure to the contaminant.

These results were consistent with those of other research work done of a similar nature (Imperiale *et al*., 2009; Lobato *et al.,* 2006; Vesna Cerkvenik *et al.*, 2001). Besides, when contaminated meat samples were subjected to different heating processes, the residue was observed to be thermally stable (Rose *et al*., 1998).

Data from single diluted 5 mL of contaminated milk diluted with 5 mL of blank milk) milk samples were compared to raw and boiled samples. Single diluted samples recorded lower ivermectin concentrations than those in corresponding raw and boiled milk samples (table 4.6). The findings showed that the mean concentrations of ivermectin residues differed significantly across raw, boiled, single-diluted samples.

The effects of additional dilution were investigated in the samples that were diluted twice (5 ml of raw milk samples diluted with 10 mL of blank). Similarly, the concentrations of raw and boiled samples changed in comparison to concentrations for corresponding double diluted samples, and thus were statistically different at $p<0.05$. Considering the results recorded, it may be deduced that at higher concentrations, the residue levels vary while at lower concentrations there is no significant difference. Therefore, dilution lowered residue concentrations in milk. The solute concentration level in the solution was highly dispersed. Similarly, there were no metabolic products of analyte degradation observed in the chromatograms obtained on the diluted samples and this ruled out presence of interfering materials. Zero signals were detected at the spiking level attributing this to the lowered concentrations that were significantly lower than the limit of quantitation (S/N 10).

It was evident that boiling and dilution do not degrade ivermectin residues in milk and to prevent occurrence, some measures have to be put in place for control purposes. The primary preventive approach can be centred towards the primary prevention of the entrance of xenobiotic substances in the form of residues into edible animal products. This approach may therefore be guided by appropriate drug use, a conventional guide for use by veterinarians and dairy as well as beef producers. To realize this, proper management of herd health is a prerequisite that also requires maintaining a clean and healthy environment on all food animals with the goal of preventing contamination of their products. Moreover, both dairy and beef farmers are advised not to utilize or even keep medications that are not approved for use in regulated drug practices (Beyene, 2016). Similarly, establishment of an effective relationship between the veterinarian-client-patient where appropriate and application of drugs with approved dosages.

Consequently, the right route of application, adherence to recommended withholding time, and identifying treated animals before the dispensation of drugs are some ways to curb the vice. Reliable maintenance of treatment records may be an excellent practice for future reference so that treated animals can be identified. Furthermore, building of a drug residue testing infrastructure capable of selecting and interpreting tests, as well as the cultivation of knowledge on proper drug use would curb presence of contaminated products in the marketplace (Beyene, 2016; Scippo *et al.*, 1994).

4.6 Susceptibility of ticks to common acaricides

The current study established two common species of ticks in the area. These were, *Rhipicephalus appendiculatus* (RA) and *Boophilus decoloratus* (BD). The tolerance results of ticks to the tested acaricides against ivermectin are given in Table 4.7. The most susceptible species to the acaricides was found to be *R. appendiculatus* with amitraz and ivermectin recording a 100% mortality on the adult ticks after seven days. Notably, 30% and 20% mortality rates were observed in cypermethrin and chlorpyrifos, respectively, whereas, 40% mortality was recorded upon combining chlorpyrifos and cypermethrin acaricides. Some level of resistance on ticks for the respective treatments was detected. However, after ovipositioning, the eggs were assessed for viability through evaluating hatchability. The hatchability after 42 days of the ticks that survived in combination, chlorpyrifos, and cypermethrin was calculated at 90%, 50%, and 40% respectively.

Furthermore, the estimated reproduction of the eggs in both the combination and cypermethrin was very low, an indication of low viability of eggs. Although chlorpyrifos had higher levels of 45.42%, it was effective for the control of *R.*

appendiculatus. *R. appendiculatus* was observed to be very susceptible to the molecules studied. Subsequently, the *B. decoloratus* species subjected to the acaricide treatment recorded a high mortality rate of 100% on ivermectin and 10%, and 20% on amitraz, cypermethrin respectively while chlorpyrifos and combined acaricides recorded 0% mortalities on adult ticks after seven days. However, the observed hatchability after 42 days of incubation was 60% for the combination and amitraz; and 80% for cypermethrin and chlorpyrifos acaricides. The levels of resistance are similarly represented graphically and are posted as Annex 10.

The results demonstrated 100% potency of amitraz and ivermectin against *R. appendiculatus,* with no significant difference noted between the two drugs employed for parasite management. Furthermore, *R. appendiculatus* indicated 6.54%, 15.60%, and 45.42% resistance levels towards a combination of cypermethrin- and chlorpyrifos, cypermethrin and chlorpyrifos acaricides respectively (Table 4.7). However, this was still within the susceptibility levels. Thus, all acaricides utilized in the study were shown to be effective in the control of *R. appendiculatus*, with amitraz and ivermectin being the most potent to the species. Subsequently, *B. decoloratus* showed susceptibility to ivermectin and a combination at 0% and 45.42% respectively. This showed that the combination was effective in managing both species. Amitraz and cypermethrin on the other hand, had efficacies of 50.18% and 65.14%, respectively. As a result, *B. decoloratus* was showing possibilities of developing resistance to the two acaricides. Further, chlorpyrifos had a percentage level of 85.75% resistance in the control of *B. decoloratus*. Hence, the efficacy of the tested acaricides that were used to suppress the parasite varied significantly.

Given that there was no great disparity in resistance, both tick species were determined to be sensitive to ivermectin, making it efficient in tick management. Secondly, despite the fact that the data for percentage tolerance for the two species differed when a combination was used, it was determined that the combination was still effective in the control of these two species. Similarly, there was a significant difference when amitraz and cypermethrin were applied and both showed efficacy against these ticks but indicated increasing resistance against *B. decoloratus* species.

Table 4. 7: Tick tolerance to synthetic acaricides and ivermectin in percentages

Treatment		***Rhipicephalus appendiculatus***	***Boophilus decoloratus***
Synthetic acaricides	Amitraz	0.00 ± 0.00^{dB}	50.18 ± 0.11^{cA}
	Cypermethrin	15.60 ± 0.06^{bB}	65.14 ± 0.16^{bA}
	Chlorpyrifos	45.42 ± 0.30^{aB}	85.75 ± 0.34^{aA}
	Combination (Chlorpyrifos and Cypermethrin)	6.54 ± 0.08^{cB}	45.42 ± 0.20^{dA}
	Ivermectin	0.00 ± 0.00^{dA}	0.00 ± 0.00^{eA}

Means followed by different small and capital letters in a column and row respectively indicate significant difference ($p < 0.05$)

Amitraz was responsive in comparison to other products and had a very rapid knock-down effect because it gets bound to and activated to adrenergic neuro-receptors in animals, inhibiting the action of monoamine oxidases (MAO) (Jonsson *et al.*, 2018). Similarly, pyrethroid acaricides have been used for long accounting for almost 25% of the global acaricide market (Casida and Quistad, 1998). Some resistance of ticks towards amitraz and synthetic pyrethroids was found in *Rhipicephalus microplus, R. decoloratus and R. appendiculutus* (Jonsson *et al.*, 2018; Abbas *et al*., 2014; Rodríguez-Vivas *et al.*, 2014; Jonsson *et al.*, 2000). In addition to the two classes of acaricides, varying levels of resistance to organophosphates (OPs) were found in the same tick population (Rodríguez-Vivas *et al*., 2014).

Many times, pyrethroids have been employed as combinations comprising two or more components. Largely, cypermethrin, a pyrethroid acaricide, is mixed with chlorpyrifos. The synergistic action of the two chemicals reduces mortality time. The combination is also indicated to have a long residual activity that increases its effectiveness as compared to amitraz or clofenvinphos (You, 2014). In another study, a similar mixture was employed to quickly eliminate all susceptible members of a targeted tick population (Novelino *et al.*, 2007). Thus, the use of acaricide combinations has been seen as an appealing strategy for postponing the development of resistance (Lovis *et al*., 2013).

Since tick-borne diseases cause major losses to farmers, it is important to control the tick vector. To this effect, several tick control strategies have been in use. Consequently, tick monitoring to detect early stages of acaricide is essential to limit resistance propagation and gather knowledge of their dispersion. In this context, studies have advised that the unique impacts of specific acaricides on specific species be utilized to identify the issue (Ghosh *et al.,* 2007).

Amongst the methods that have been in use in quashing tick populations in the developing world (FAO, 2004; Regassa, 2000) are chemotherapeutic tick control programs as the primary methods (Bianchi *et al*., 2003). A progressive declining trend in the acaricidal drugs' efficacy has however threatened this method through the emergence of resistance (Chardonnet *et al.*, 1994). Hence, it would be imperative to devise an approach for prolonging the effectiveness of currently available acaricides (Abbas *et al*., 2014). Similarly, newer methodologies of combining chemistry and

computational biology with efficient target screening might lead to the generation of newer generation acaricides (Ghosh *et al.*, 2007).

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Consequently, the following conclusions were deduced from this study;

i. Ivermectin residues were present in milk samples obtained directly from farmers and vendors. The concentration levels did not agree with the recommended zero MRL in milk.

ii. Ivermectin residues were not present in milk after the 18th day. Ivermectin residues were observed to be excreted through milk 12 hours later after treatment and persisted for 17 days. The highest levels were observed on the 2nd day after which levels began to reduce until day 17.

iii. Boiling milk containing ivermectin residues did not degrade the residue since the molecule remained quite stable. Similarly, diluted samples presented quantifiable levels of the drug residue. Subsequently, boiling does not eliminate veterinary drug residues in food, as earlier reported

iv. *R. appendiculutus* recorded susceptibility to the four acaricides tested against while emerging resistance was observed on *B. Decoloratus* towards amitraz, chlorpyrifos, and cypermethrin. However, both species were susceptible to combined acaricide that had chlorpyrifos and cypermethrin.

5.2 Recommendations

5.2.1 Recommendations based on the study

The following are the recommendations from the study:

i. Replace use of IVM with safer effective macrocyclic lactones that are effective against endectocides in lactating cows to eliminate risk of IVM excretion in milk

ii. The withdrawal duration for ivermectin should be set at the 18th day after administration to ensure that milk is safe for human consumption and devoid of IVM contamination, even in events of off-label use.

iii. Seek for other alternative milk processing techniques instead of boiling and dilution to reduce and eliminate IVM concentrations in milk

iv. The combined (chlorpyrifos and cypermethrin) acaricide can be used as an effective method for the control of ticks in the area.

5.2.2 Recommendations for further study

The study recommends further studies to;

i. Evaluate whether ivermectin residues persist in other dairy products like cheese and yoghurt and other animal products including beef, liver, blood, and edible interior parts of animals.

ii. Establish *in-vivo* effectiveness of the acaricides to compare how well they work under field conditions, in comparison to the *in-vitro* tests carried out in this study.

iii. Establish the mechanism of resistance of *B. Decoloratus* against acaricides.

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ANNEXURES

Annex 1: Questionnaire

Evaluation of farmers tick control practises

	Farmer No: _____			
	Date: _____			
	Farm Information			
1	Number of animals in the farm:	Total _____	Adults _____	Young_____
2	Total number of cows in the herd:	_____		
3	Number of lactating cows:	_____		
4	Average amount of milk produced in litres	_____		
5	Amount of milk consumed within the farm	_____		
6	Amount of milk sold	_____		
7	Grazing Methods:	Zero grazing ☐	Herding ☐	Rotational grazing ☐
8	Do animals get infested with ticks?	Yes ☐	No ☐	
9	What mode of tick control is used in the farm?	Chemical method ☐	Others (Specify)	_____
10	If chemical method, which mode?	Injection ☐	Dipping ☐	Spraying ☐
11	If injection, which drug?	Ivermectin ☐	Eprinomectin ☐	Others ☐
12	If ivermectin, how often?	_____		
13	If acaricide is used, which one?	_____		
14	Is the method used in tick control effective?	Chemical method	Yes ☐	No ☐
		Injection	Yes ☐	No ☐
		Others (Specify)	Yes ☐	No ☐
17	Do they consult animal health practitioners to provide treatments?	Yes ☐	No ☐	
18	If no, who treats the animals?	_____		

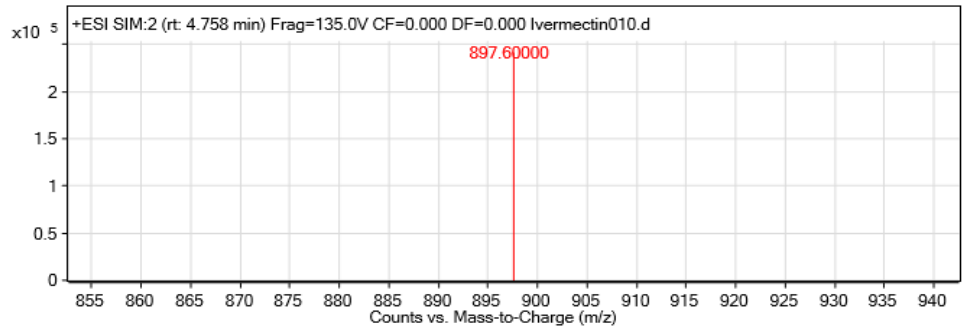
DECLARATION, Any information generated through this questionnaire shall be used only for the purposes of this study. The information shall also remain confidential to the researcher, and the farmers not be held liable to any information generated.

Researcher's Signature: _____ Date: _____

Annex 2: Peak abundances at varied fragmentor voltages

a. Peak abundances at fragmentor voltage of 135

Fragmentor Voltage	Collision Energy	Ionization Mode
135	0	ESI

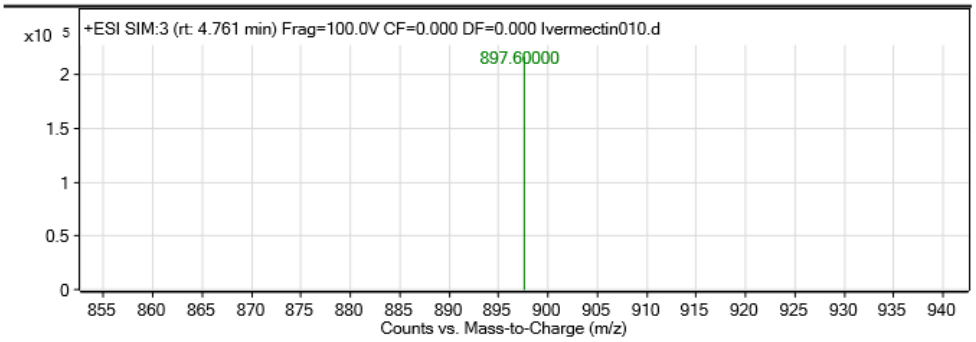


Peak List

m/z	Abund
897.6	240995.67

b. Peak abundances at fragmentor voltage of 100

Qualitative Analysis Report

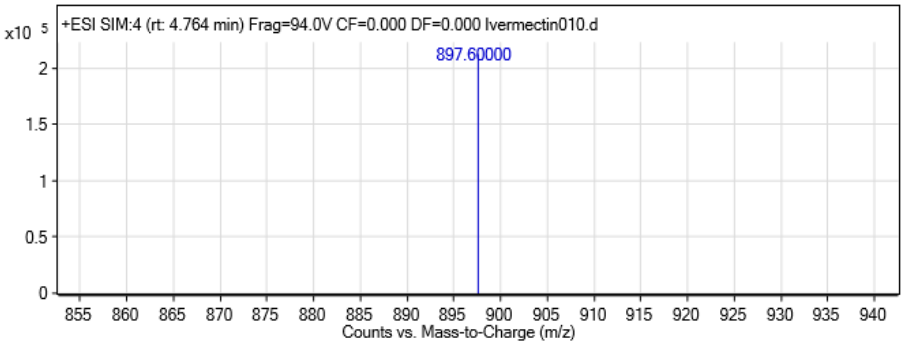


Peak List

m/z	Abund
897.6	216714.7

c. Peak abundances at fragmentor voltage of 94

Fragmentor Voltage	Collision Energy	Ionization Mode
94	0	ESI

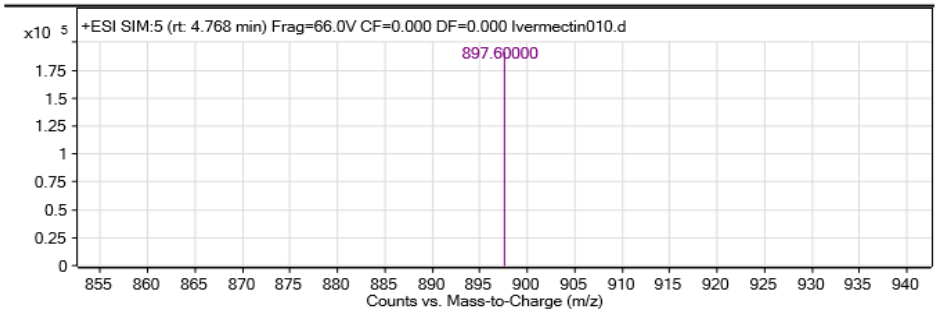


Peak List

m/z	Abund
897.6	212232.5

d. Peak abundances at fragmentor voltage of 66

Qualitative Analysis Report

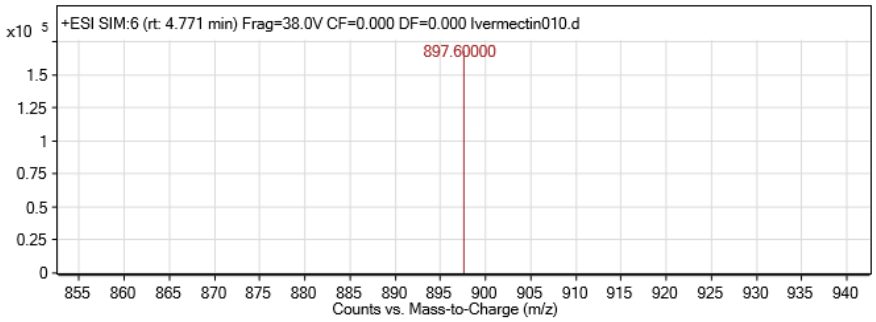


Peak List

m/z	Abund
897.6	190757.38

e. Peak abundances at fragmentor voltage of 38

Fragmentor Voltage	Collision Energy	Ionization Mode
38	0	ESI

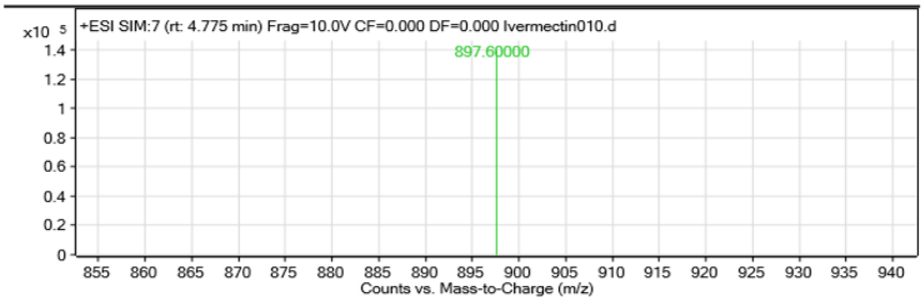


Peak List

m/z	Abund
897.6	167599.61

f. Peak abundances at fragmentor voltage of 10

Qualitative Analysis Report

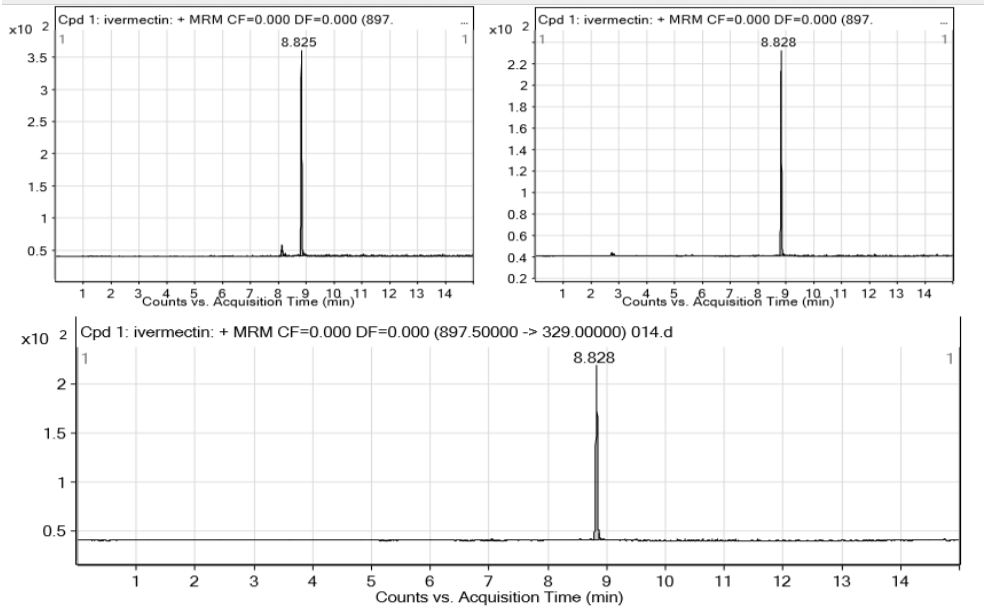


Peak List

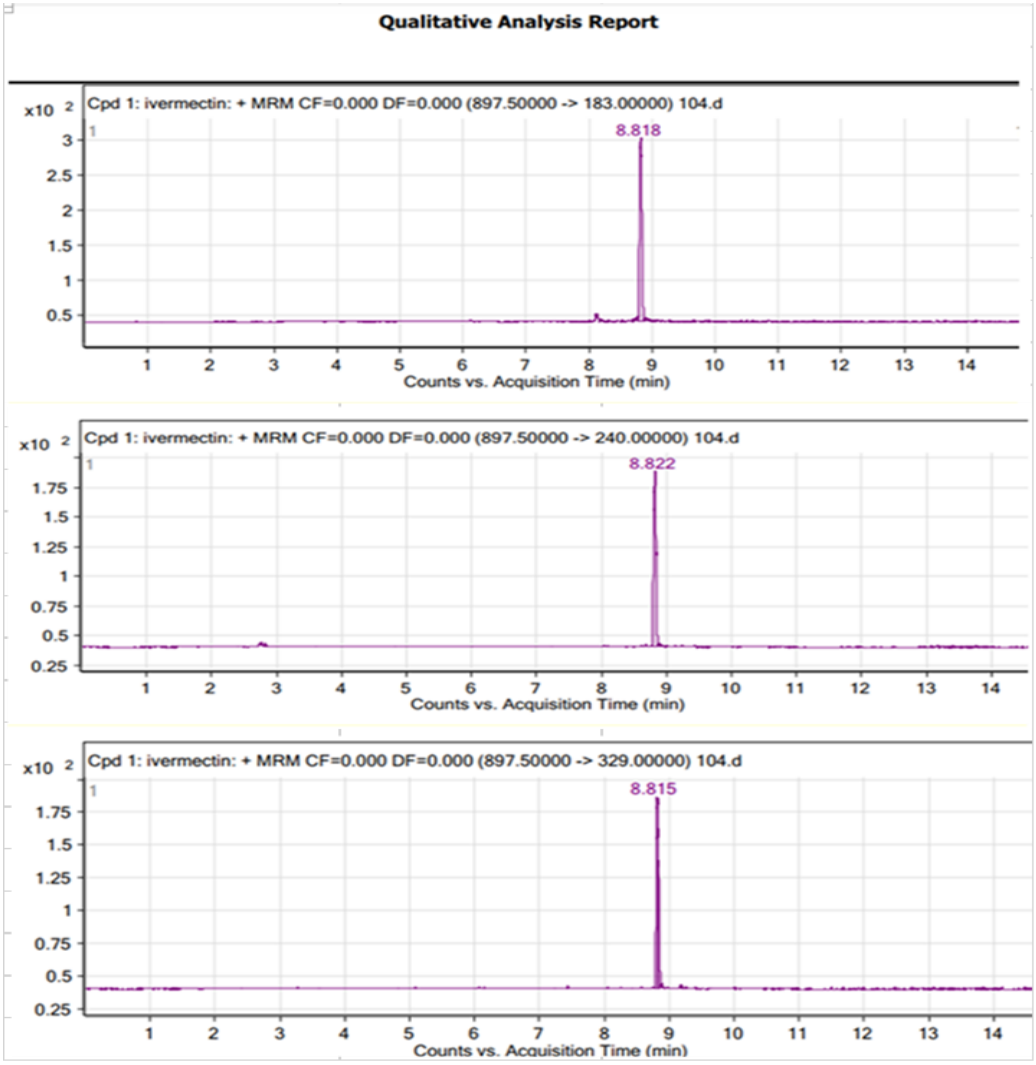
m/z	Abund
897.6	139108.23

--- End Of Report ---

Annex 3: Retention times for Ivermectin



Annex 4: Chromatograms for product ions



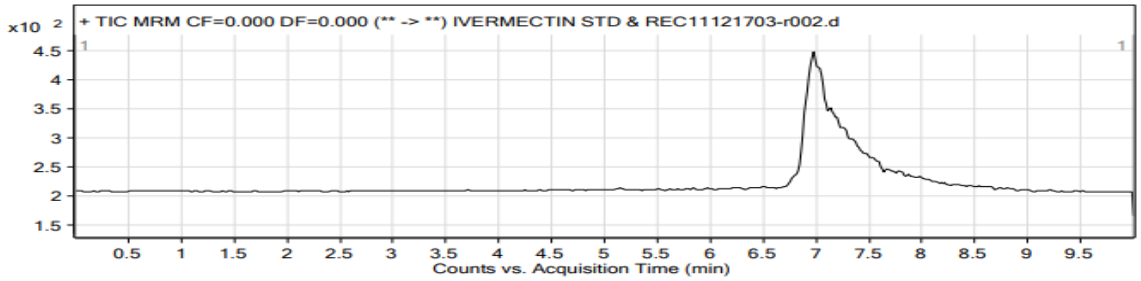
Annex 5: Peaks of 5ppb standard for 5 mM ammonium formate in water and 0.1% formic acid in water (A) and 0.1 % formic acid in methanol (20:80)

Qualitative Analysis Report

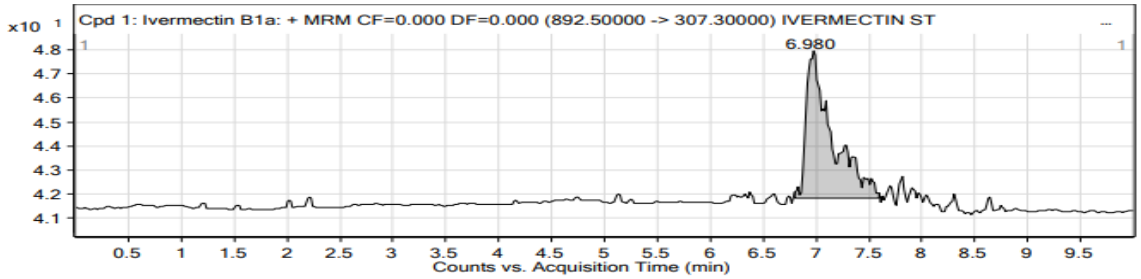
Data File	IVERMECTIN STD & REC11121703-r002.d	**Sample Name**	IVERMECTIN STD5PPB 111217
Sample Type	Calibration	**Position**	P1-A3
Instrument Name	DVS LC-QQQ	**User Name**	
Acq Method		**Acquired Time**	12/11/2017 2:47:39 PM
IRM Calibration Status	Not Applicable	**DA Method**	07102020 pesctcide mix MRM.m
Comment			
Sample Group		**Info.**	
Stream Name	LC 1	**Acquisition SW Version**	6400 Series Triple Quadrupole B.08.00 (B8023.0)

User Chromatograms

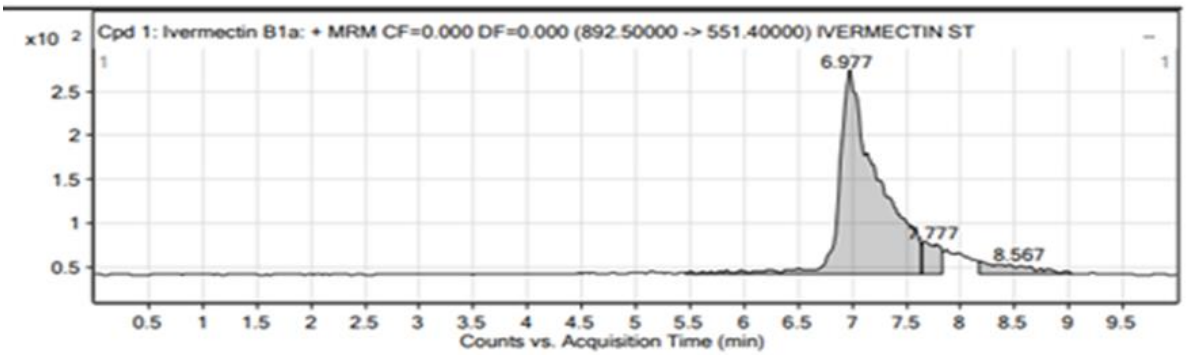
Fragmentor Voltage 0 **Collision Energy** 0 **Ionization Mode** ESI

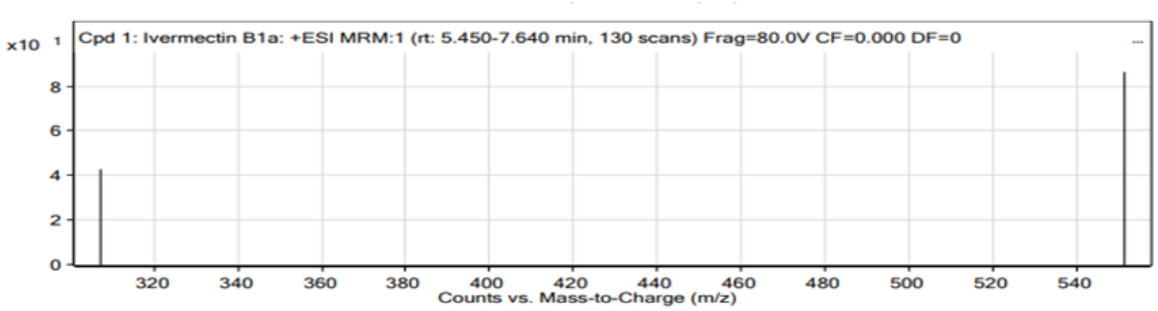


Compounds



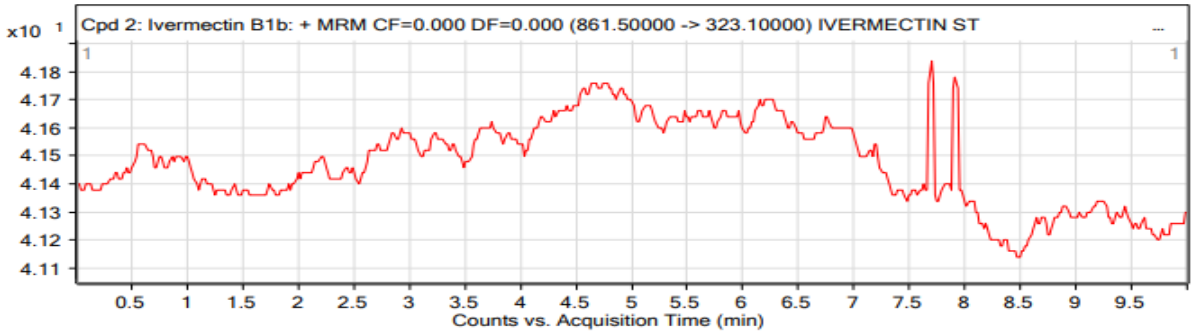
Qualitative Analysis Report



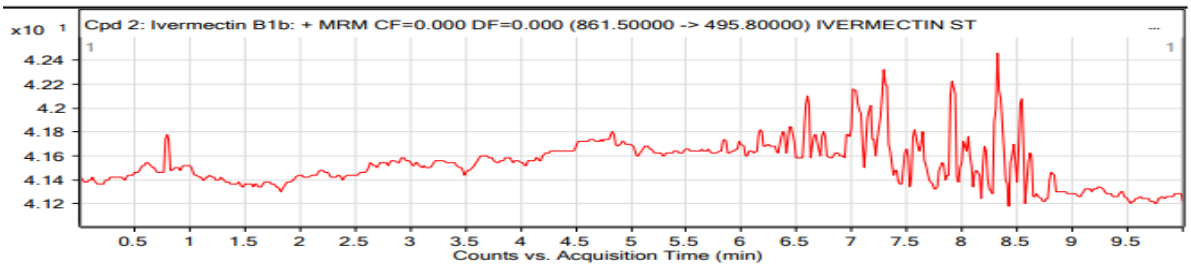


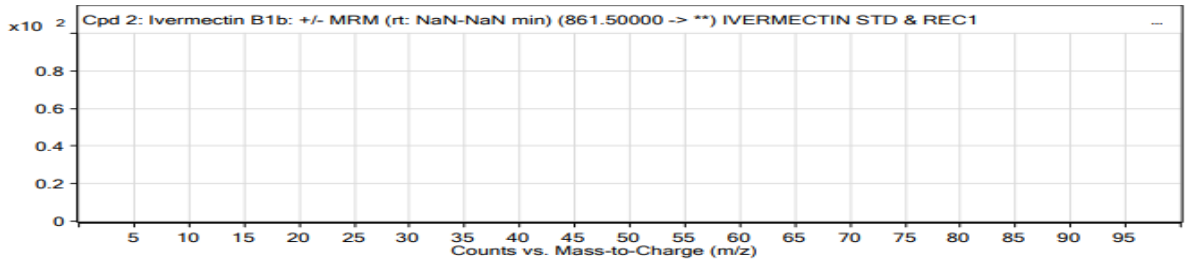
Peak List

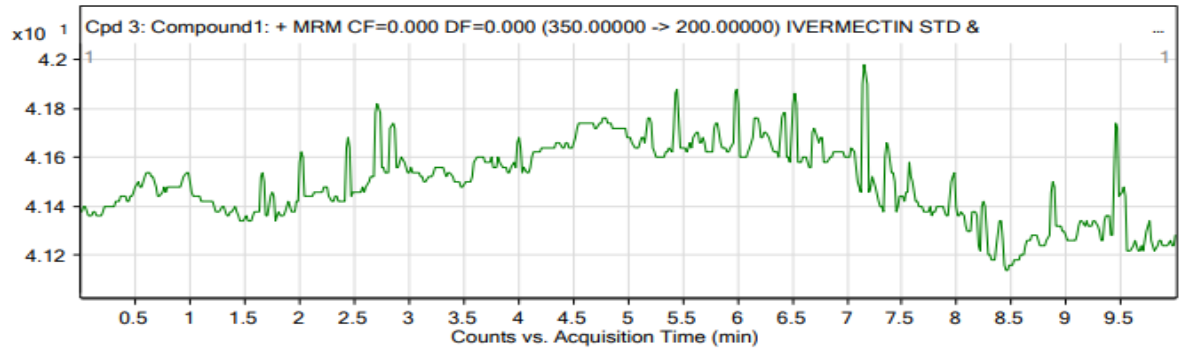
m/z	Abund
307.3	42.54
551.4	86.06



Qualitative Analysis Report







- End Of Report ---

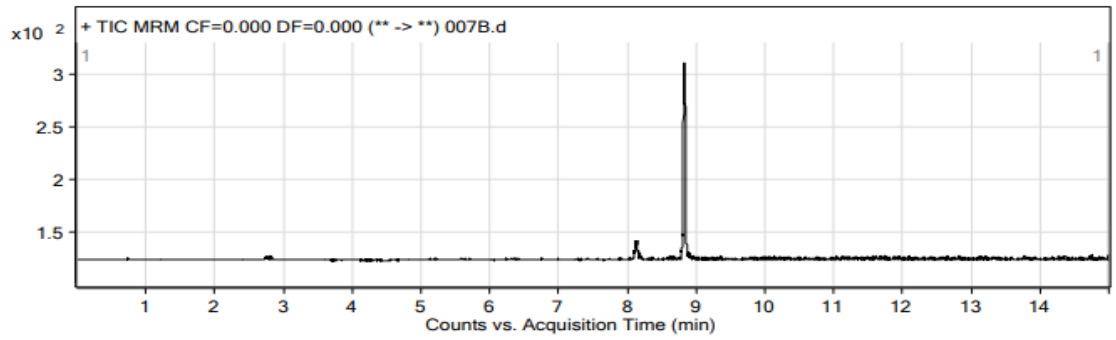
Annex 6: Peaks of 5ppb standard for 0.1% formic acid in water (A) and in methanol (B) in an isocratic flow

Qualitative Analysis Report

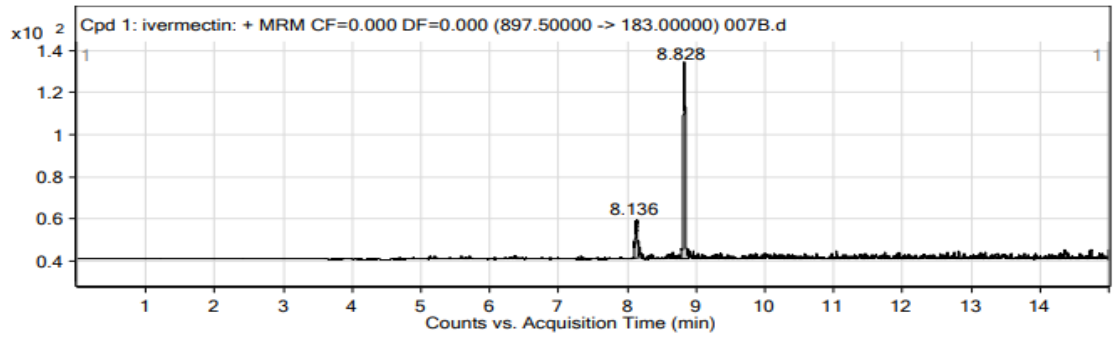
Data File	007B.d	**Sample Name**	Ivermectine STD 5ppb
Sample Type	Calibration	**Position**	P1-A3
Instrument Name	DVS LC-QQQ	**User Name**	
Acq Method	21082020 Ivermectin MRM.m	**Acquired Time**	8/21/2020 6:16:14 PM
IRM Calibration Status	Not Applicable	**DA Method**	07102020 pesctcide mix MRM.m
Comment			
Sample Group		**Info.**	
Stream Name	LC 1	**Acquisition SW Version**	6400 Series Triple Quadrupole B.08.00 (B8023.0)

User Chromatograms

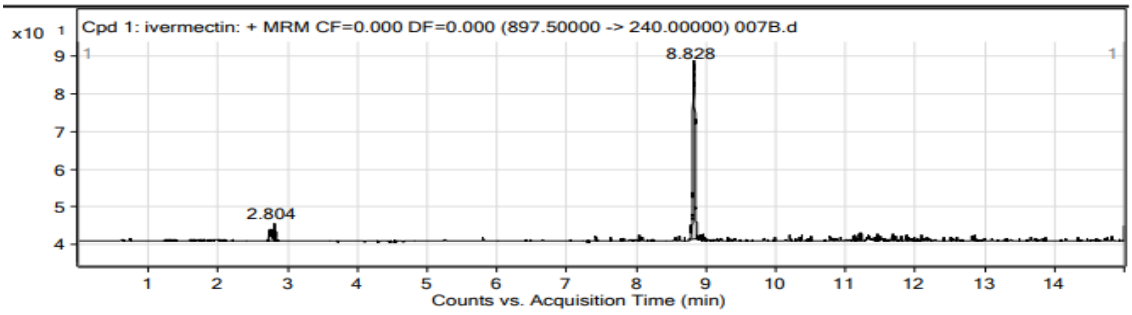
Fragmentor Voltage 150 **Collision Energy** 68 **Ionization Mode** ESI

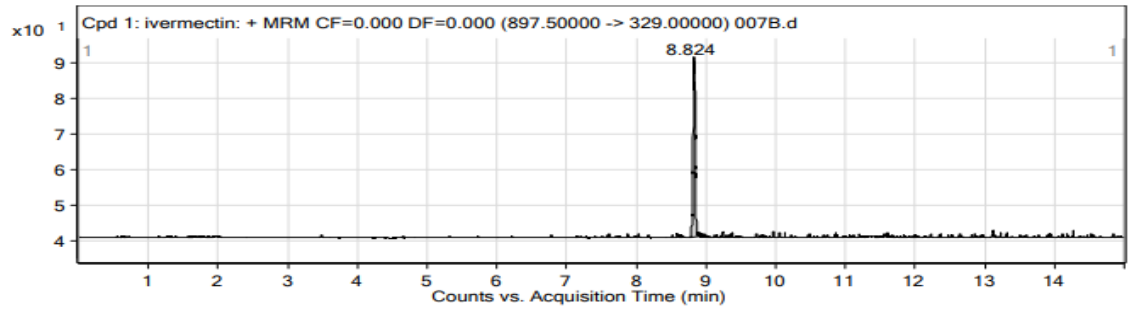


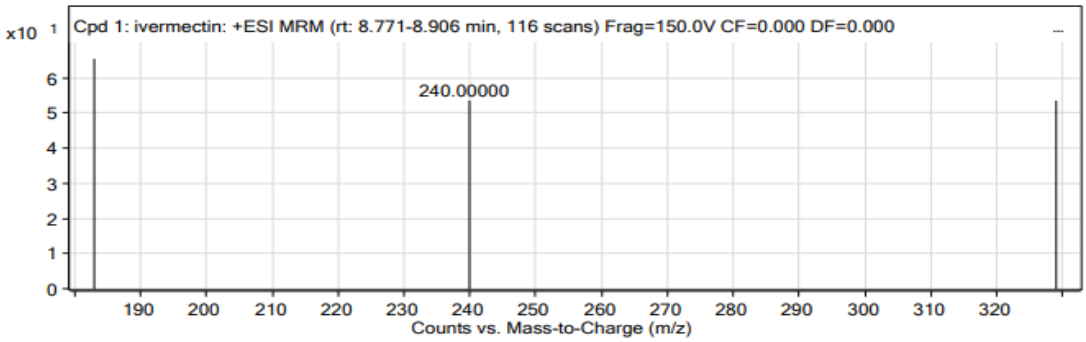
Compounds



Qualitative Analysis Report







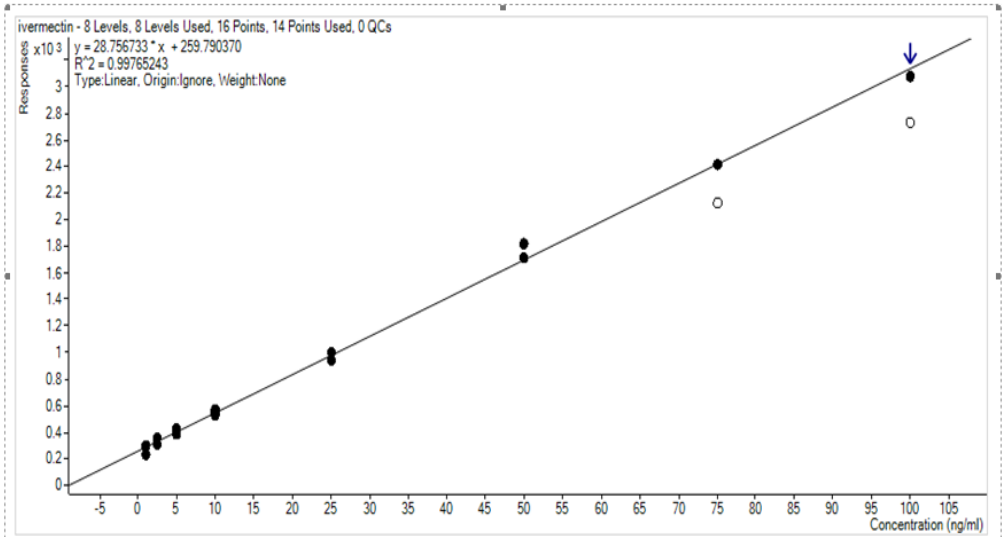
Peak List

m/z	Abund
183	65.45
240	53.55
329	53.58

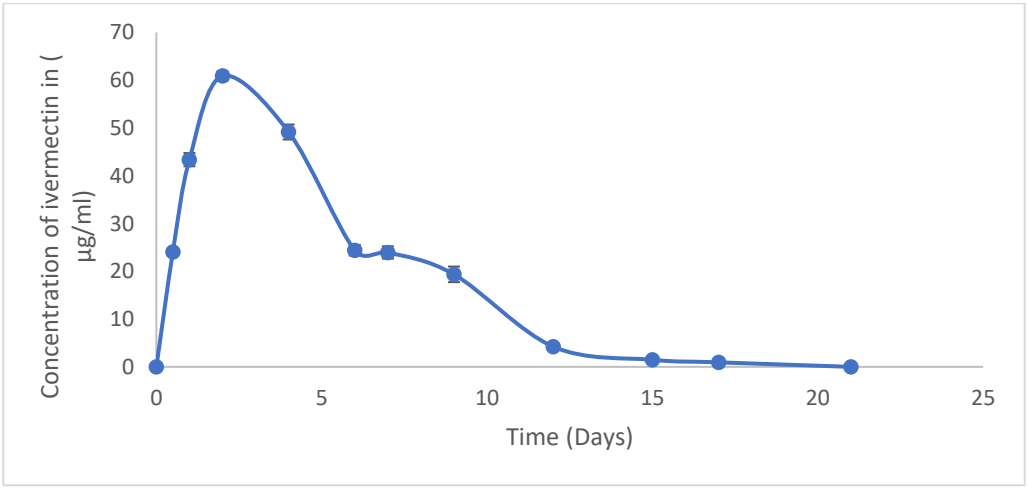
--- End Of Report ---

Agilent Technologies

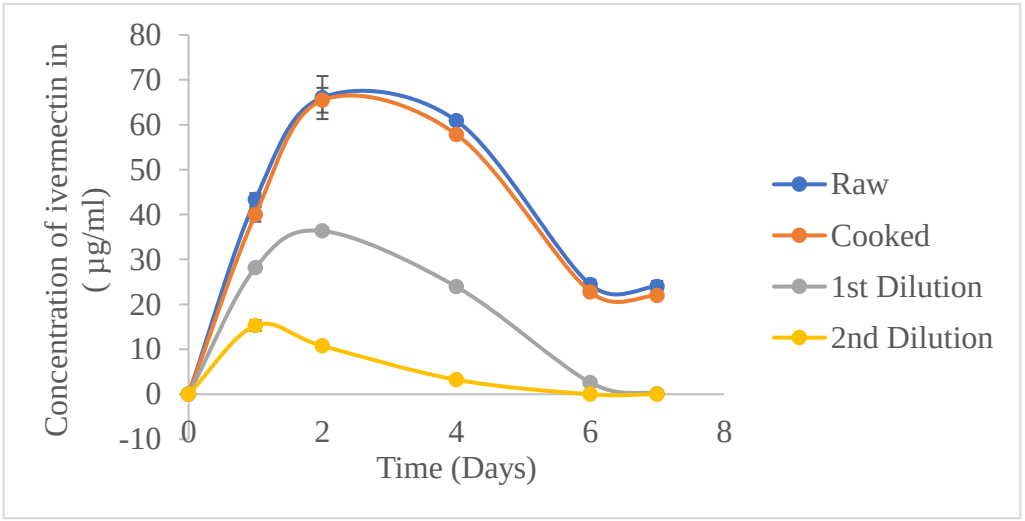
Annex 7: Solvent calibration curve



Annex 8: Concentration levels of ivermectin in milk from treated dairy cow.



Annex 9: Graph representation for concentration levels of ivermectin residues after boiling and dilution milk



Annex 10: Percentage resistance of ticks subjected to acaricides and ivermectin