DITHIOCARBAMATES AND ETHYLENETHIOUREA RESIDUE LEVELS IN TOMATO AND SWEET PEPPER FROM KIRINYAGA AND NAIROBI COUNTIES

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FEBRUARY, 2020

DECLARATION

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

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DEDICATION

This work is dedicated to my son Alvin Karanja and my daughter Belinda Nyaguthii.

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ABBREVIATIONS AND ACRONYMS

ADI	Acceptable daily intake
AI	Active ingredient
BDL	Below detection limit
DAD	Diode array detector
DTC	Dithiocarbamates
EBDC	Ethylenebis-dithiocarbamate
EC_{50}	Effective concentration on 50% test animals
EPA	Environmental protection agency
ETU	Ethylenethiourea
FAO	Food and agricultural organization
GC	Gas chromatography
HPLC	High pressure liquid chromatography
JMPR	Joint meeting on pesticide residues
LC_{50}	Lethal concentration that kills 50% cases
LD_{50}	Lethal dose that kills 50% of test animals
LOB	Limit of blank
LOC	Level of concern
LOD	Limit of detection
LOQ	Limit of quantification
MRL	Maximum residue level
MS	Mass spectrometry
ND	Not detected
PBDC	Propylenebis-dithiocarbamate
PHI	Post harvest interval
UV	Ultra violet
WHO	World Health Organization

ABSTRACT

Vegetables are a major source of essential nutrients such as minerals, proteins, energy and roughage. Some vegetables such as tomato and sweet pepper can be eaten raw or cooked. These vegetables are affected by pests and diseases that are controlled using pesticides among which are fungicides such as dithiocarbamates (DTCs) that are toxic to human and animals at high concentrations. DTCs are commonly used in different combinations in Kenya and one of their metabolites, ethylenethiourea (ETU) a carcinogenic compound, has a long residual time of between five to ten weeks. Cooking degrades DTCs to metabolites while proper cleaning can remove the residue considerably. This study investigated the levels of dithiocarbamate fungicides propineb and mancozeb, and the metabolite ETU in tomato (*Lycopersicon esculentum mill*) and sweet pepper (*Capsicum annum l)* during wet and dry seasons and also after different temperature and cleaning treatments. The analyte were extracted from vegetables using acetonitrile-dichloromethane-chloroform mixture and analyzed using HPLC. The results obtained showed residue levels of propineb and mancozeb in tomato which were significantly higher in the wet season in three out of the four markets. The levels of mancozeb in tomato were ranging from 2.56±0.12 mg/kg in the wet season to below detection limit (BDL) in the dry season while in sweet pepper the levels ranged from 2.69±0.57 mg/kg in wet season to 0.16±0.00 mg/kg in dry season. Propineb levels ranged from 3.97±0.50 mg/kg in wet season to BDL in dry season in tomato while in sweet pepper the range was 6.54±0.72 mg/kg in wet season to BDL in dry season. The ETU levels in tomato samples were significantly higher in dry season than wet season and ranged from 27.94±0.39 mg/kg to BDL in wet season while in sweet pepper the levels ranged from 8.88±1.55 mg/kg in dry season to BDL in wet season. Two out of four markets had propineb residues in tomato above maximum residue limit (MRL) set by WHO/FAO and EU of 3 mg/kg while there were no levels of mancozeb above MRL. ETU mean residue levels exceeding the MRL of 0.05 mg/kg were noted in all vegetable samples. The temperature treatment showed a significant increase of ETU residues from a low mean of 9.43±0.03 mg/kg at 25℃ to a mean of 12.43±0.38 mg/kg at 90 ℃ showing an increase of ETU residues with cooking. Cleaning with sodium hypochlorite (chlorine water) showed a reduction of ETU residue by 99.9% in tomato and by 100% in sweet pepper. The mean residue levels of ETU in tomato reduced from a mean of 46±0.71 mg/kg to 0.05± 0.00 mg/kg while in sweet pepper a reduction from 2.14±0.02 mg/kg to BDL. Cleaning with water and chlorine water differed significantly in removing the ETU residue. Mancozeb mean residue levels in tomato cleaned with sodium hypochlorite reduced by 95.2% while propineb reduced by 80.4%. Cleaning with sodium hypochlorite showed a significant removal of fungicides than water only. The result from this study indicates high residue levels of mancozeb, propineb and ethylenethiourea in fresh and cooked tomato and sweet pepper. There is therefore a need for cleaning with chlorine water and rinsing with water before consumption and a regular surveillance of the fungicides and metabolite.

CHAPTER ONE

INTRODUCTION

1.1 Background

Vegetable production is one of the major branches of horticulture. Vegetables are considered as an asset providing income to the growers and providing a vital part of human diet. In Kenya vegetables which contain minerals, vitamins and essential amino acids are used in common foods. Vegetables such as tomatoes and sweet pepper are eaten raw or cooked as the fruit part. They are usually affected by fungal infections and other pests, and hence are usually sprayed for protection or for curative purpose using various pesticides.

Pesticides include insecticides, fungicides and herbicides among others. The term pesticide is associated with materials intended to kill, control or repel pests. In contemporary agriculture over 30% of the produce does not reach the consumers market, and without use of chemical agents the loss would be doubled (Gupta and Thind. 2006). It has also been noted that without use of pesticides crop yield could drop by as much as a third and food prices can increase by as much as 75% (Yadav., 2010).

Fungicides are chemical compounds or living biological organisms used to prevent growth of fungal spores or kill them. They destroy or prevent diseases like late bright, early bright and leaf spot. Dithiocarbamates are a group of fungicides used to kill fungi and their use is not restricted. Dithiocarbamates have thyroid effect, are neurotoxic, mutagenic and teratogenic hence suspected to have carcinogenic effects (FAO/WHO., 2012). In Kenya dithiocarbamates are sold in different formulations used in different percentages from a study done in Kirinyaga County as shown in Table 1.

Table 1: Percentage use of some pesticides in Kirinyaga County

PESTICIDE	CATEGORY	% USE
Alphacypermethrin	Insecticide	75.0
Mancozeb	Fungicide	75.0
Propineb/ cymoxanil	Fungicide	28.3
Sulphur	Fungicide	28.3
Metalaxyl/mancozeb	Fungicide	12.5
Copper oxychloride (green copper)	Fungicide	10
Cuprous oxide (red copper)	Fungicide	5.8
Copper hydroxide (green copper)	Fungicide	0.8

Source: Waiganjo *et al.* (2006)

From Table 1, Mancozeb had a high percentage use in Kirinyaga County at 75% while propineb is only at 28.3%. The other fungicide formulations are used in moderation. Ethylenethiourea (ETU) which is a metabolite of fungicides such as mancozeb, zineb, metiram and maneb has a half-life of 1-7 days in the open environment and between 5-10 weeks in the soil (Shukla *et al.*, 2001).

The residue of dithiocarbamate fungicides is converted to ethylenethiourea (ETU) during storage; while foods cooked containing their residues decompose increasing levels of ethylenethiourea. Thermal treatment of the dithiocarbamate maneb showed conversion to ETU by 28% at 100 ºC and 32% at 121 ºC (Kontou *et al.*, 2004). ETU has been found in human diet exposed to dithiocarbamate fungicides and vegetables obtained from markets when consumed may have residues of these fungicides and their metabolites which can be harmful to consumers hence need for this study. Studies have shown that ethylenebisdithiocarbamates (EBDC) are in the category of pesticides whose residues are more frequently found in raw agricultural products (Kontou *et al*., 2004). In Kenya a study carried out in some markets found ethylenethiourea residues ranging from a mean of 17.5±0.1 to 10.6±0.8 mg/kg in tomatoes (Kariuki. 2008). A surveillance study done on dithiocarbamate residues in different vegetables from markets in Valencia Spain showed that 96.9% of pepper and 87.5% of tomato samples had positive results (Lopez *et al*., 2012). ETU residue levels have been found in various fruit juices in Turkey at values ranging from 0.08 to 0.11 mg/kg (Gul and Buket. 2008). In Bukavu District, Congo residue levels of mancozeb ranged from below detection level (BDL) to 4.65 mg/kg with 24% of tomato samples having residue levels above maximum allowed residue levels (MRL) set by European Union (EU) of 3mg/kg (Mpiana *et al.*, 2014).

Cleaning vegetables with water and other processing methods of vegetables is known to affect the residue levels of pesticides. Studies of cleaning with chemicals like basic sodium hypochlorite (chlorine) have shown a reduction of the residues of EBDCs and ETU considerably (Hwang *et al*., 2002).

Figure 1.1: Sweet pepper vegetable with visible pesticide residue
(Source: self photo)

1.2 Statement of the problem

The awareness on the importance of vegetables among Kenyans has enhanced the consumption of horticultural products such as tomato and sweet pepper, consumed either raw or cooked. This has increased the production of these vegetables that involve high use of fungicides such as dithiocarbamates. The fungicides are known to resist immediate degradation or produce metabolites which are stable. The Kenya Bureau of Standards (KEBS) and international bodies like World Health Organization (WHO) are mandated to set maximum residue levels on vegetables based on the recommended application levels. However, farmers usually increase the application rates of pesticide to ensure their produce reaches the market in good quality (Mugambi. 2009). During the wet seasons a lot of fungicide is applied to cater for increased fungal infestation, while during dry seasons some fungicides may degrade due to high temperatures. This study therefore investigated levels of the residues of dithiocarbamates and ethylenethiourea in fresh vegetables consumed in different seasons and markets in Kenya.

Vegetables sold by some vendors when already cut, may pose danger to consumers since they are not usually properly cleaned and may contain high residues above the recommended levels. Some of these residues may also decompose to more harmful metabolites on cooking. There is lack of information from literature on the effect of cooking and washing on ethylenebisdithiocarbamates and ethylenethiourea residue levels especially in vegetables such as tomato and sweet pepper. There is also a need to study levels of mancozeb and ethylenethiourea (ETU) in different types of vegetables, in different seasons, and processing then provide a method on how to reduce the residues of the analyte to the legal and healthy limits.

1.3 Justification

Fungicides such as dithiocarbamates and their metabolites have been shown to cause cancer and high residue levels of the fungicides are injurious to the environment, birds and fish. In Kenya cancer is ranked third as a cause of death, which is 7% of total mortality yearly (MPH/MMS., 2011). Globally there are over 18.1 million new cases of cancer with over 9.6 million deaths translating to over 13% of total deaths (Freddie *et al,* 2018). Some of the cancer cases may arise from diets containing carcinogenic compounds like ethylenethiourea (ETU) whose residue levels are known to increase in cooking while decreasing considerably with proper cleaning or processing. Monitoring the levels of pesticides residue in vegetables, during different seasonal conditions and different processing conditions such as cooking and cleaning treatments will inform consumer safety on food consumed.

1.4 Hypothesis

Residue levels of dithiocarbamates and ethylenethiourea (ETU) on vegetables are not dependent on growing season and are not reduced by heating and cleaning.

1.5 Objectives of the study

1.5.1 General objective

To investigate residue levels of dithiocarbamates and ethylenethiourea in selected vegetables in dry and wet seasons, different temperature and cleaning treatment conditions.

1.5.2 Specific objectives

(i) To determine the residue levels of propineb, mancozeb and ethylenethiourea (ETU) in tomato and sweet pepper from markets in Nairobi and Kirinyaga counties during both dry and wet seasons.

(ii) To determine the effect of different temperatures on levels of mancozeb and ETU in tomato and sweet pepper obtained from a farm in Kirinyaga County.

(iii) To determine the effect of different cleaning methods on levels of propineb, mancozeb and ETU in tomato obtained from a farm in Kirinyaga County.

1.6 Significance of the study

In Kenya,cancer has become a very serious killer disease. Ethylenethiourea, a metabolite of ethylenebisdithiocarbamates fungicides is known to cause cancer and has a very long half- life and residual time of up to 70 days (10 weeks). This requires that vegetables used extensively have the residue levels monitored in different seasons, markets and temperatures and are properly processed before selling to remove harmful pesticide residues. Therefore, this study will give the status of residue levels of dithiocarbamates and ethylenethiourea in tomatoes and sweet peppers consumed in Kenyan market. The study will further provide information on effect of temperature and different processing methods on the residue levels using a simultaneous method of extraction and analysis.

1.7 Scope and limitation of the study

This study deals with only two vegetable crops, tomato (*Lycopersicon esculentum Mill*) and sweet pepper (*Capsiccum annum L*) although there are many vegetables consumed by Kenyans. Only propineb, mancozeb and ethylenethiourea (ETU) (a metabolite of mancozeb) were analyzed although there are many fungicides and metabolites. This study focuses the two dithiocarbamates propineb and mancozeb with its metabolite ETU although there are many metabolites, because the fungicides are broadly used in Kenya and ETU is the more hazardous metabolite.

The effect of temperature on the conversion of ethylenebisdithiocarbamates to ethylenethiourea is only done on mancozeb because it degrades to ETU while propineb degrades to propylenethiourea (PTU) which is not considered in this study because it is not very harmful and propineb formulations are less used in Kenya when compared to mancozeb formulations. The investigation on effects of different methods of cleaning and temperature treatments only used samples from a farm with the gardens set up in this study to provide samples sprayed with the different fungicides under study. The study on seasonal variation of residues only used samples from markets in Nairobi and Kirinyaga Counties.

The study had some limitations which included the following. The study did not consider post harvest interval (PHI) used by the farmer before selling, and length of stay of the produce on transit and in the markets. Another limitation was that there was no control on the chemical composition of fungicides used by farmers, spray mix or spray mixing order. The study was also limited in that horticultural crops require enough water and hence type of watering or irrigation was not considered. The other limitation was that there was no consideration on any processing by vendors and farmers before selling which may considerably reduce or affect residue levels of study analyses.

CHAPTER TWO

LITERATURE REVIEW

2.1 Vegetable farming

Vegetables such as tomato and sweet pepper are protective foods which provide minerals, vitamins, proteins and energy to the human body which help to overcome some health disorders such as colds, scurvy, anemia and others. In Kenya sweet pepper and tomato are grown in horticultural growing areas like Kirinyaga County, Subukia area of Nakuru County, Meru, Isiolo and Nyandarua Counties. Kirinyaga County is one of the major horticultural producing zones supplying Nairobi (Kavoi *et al.,* 2004).

2.1.1 Pepper (*Capsicum annum L*)

Pepper is from the *Solanaceae* family with many names such as sweet pepper, bell pepper, chinense pepper, aromatic pepper, bonnet pepper and in *Kiswahili* language as *piipili kali* (hot pepper) or *pilipili hoho* (sweet pepper). Capsicum originated in Central and South America where up to date there are about 25 species in the wild. Domestication for cultivation started about 6000 years ago in Mexico (Kraig *et al.*,2014).

In modern agriculture the different species of *Capsicum* have been cross-bred extensively. In Europe sweet pepper is grown mainly in green houses and also in open fields during summer.Different types of Pepper are used in sauces and appetizers and also as a vegetable of which there is no clear distinction. Hot pepper is used in traditional medicines in Africa and other parts of the world. The sharp taste in pepper is mainly from active ingredient or chemical Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide). The compound leads to an elevated release of mucous from mouth, stomach and bowel causing peristalsis. It has also shown anti-oxidant, anti-mutagenic, anti-carcinogenic, immunosuppressive activities and antibiotic abilities by inhibiting bacterial growth and platelet aggregation (Grubben *et al.*, 2004).

Green sweet pepper is known to contain about 86% water, 2% proteins, 0.8% fat, 10.3% carbohydrate and 2.6% fiber. An amount of 100 grams of sweet pepper contains 29 mg calcium, 61 mg phosphorous 2.6 mg iron, 180 µg beta carotene (red mature-2760 µg), 0.12 mg thiamin, 0.15 mg riboflavin, 2.2 mg niacin and 140 mg ascorbic acid (Grubben *et al.*, 2004). *Capsicum* fruits contain over 100 compounds contributing to the flavor and aroma, however the typical taste and smell of sweet pepper is as a result of a volatile compound known as 2-methoxy-3-isobutylpyrazine ($C_9H_{14}N_2O$). The world production of capsicum peppers stood at 34.5 million tons in 2016 according to food and agricultural organization (FAO) statistics.

In Kenya capsicum can grow in highland and low land at conditions of between 18 ºC to 30 ºC, however sweet pepper does well in cooler areas. The plant can grow in different types of soils but is most suited to a well drained sandy or loamy soil with a pH of 5.5 to 6.8. Pepper is affected by many pests and diseases including fungal diseases such as damping off caused by *Pythium aphanidermatum*, *Fusarium* and *Sclerotium.* Powdery mildew also known as *Leveillula taurica* is common during the cool seasons in Kenya. *Anthracnose* or ripe fruit rot caused by *Colletotrichum capsici* to a lesser degree and mainly *Colletotrichum leosporioides* , velvet spot *Cerscospora unamunoi* which is known to cause defoliation. *Phyptophathora* bright affects the entire plant parts and is caused by *Phyptophathora capsici.* A fungicide such as Mancozeb (Dithane M45 75 WP) has been found effective against ripe fruit rot (Gupta and Thind., 2006). Other dithiocarbamates like propineb are also used.

2.1.2 Tomato *(Lycopersicon esculentum Mill*)

Tomato is a member of the *Solanaceae* family which originated from South America and was domesticated in Mexico (Maharaj and Autar, 2007). The plant is also known as *tomate* in French and *nyanya* in *Kiswahili* language. Tomato is a highly valued horticultural crop used in salads, in sauces, in processed forms such as tomato sauces, juices, canned fruits and diced fruits and also as a flavoring in different foods (Olaniyi *et al.*, 2010).

Tomato contains 93.1% water when mature and ripe while its seed has 24% of edible oil. An amount of 100 grams contain 0.64 mg carotene, 0.09 mg thiamin, 0.01 mg riboflavin, 1.0 mg niacin, 17 µg folate, 17 mg ascorbic acid, 0.1 mg Zinc, 0.5 mg iron , 24 mg Phosphorous,7 mg Magnesium, 7mg calcium, 3.1 mg carbohydrates ,0.3 g fat , and 0.7 g proteins among other components which contribute to the taste and aroma (Grubben *et al*., 2004). Lycopene (E160-d) is the dominant carrotenoid giving tomatoes its red color but Beta-carotene is also available while unripe tomatoes have an alkaloid tomatine. Lycopene is known to contribute towards reduction of carcinogenic substances due to its anti-oxidant properties (AVDRL., 2003).

Tomato grows well in well drained loamy, sandy- loamy to clay-loamy with pH ranging from 6.0 to 7.0. The optimum temperature for growth is between 20 ºC to 27 ºC. In Kenya the crop is grown by small scale farmers ranging from a few stems to 6 acres on the higher side. The 2004 production for central Kenya was 75,101 tons with a market value of Kenya shillings 1billion (KARI. 2005). The 2013 national production was 400,204 tons with a market value of Kenya shillings 11.8 billion in a farm area of 24,074 hectares (HCDA. 2015).

There are various modern varieties including hybrids and all are affected by various pests and fungal diseases such as early and late bright, leaf spot (*Septoria lycopersici*), powdery mildew (*Leveillula taurica*) , root rot; (*Phyptophthora parasitica* and *Phypyothora capsici*)among others. Late bright (*Phytopthora infestans*), early bright (*Alternaria Solani*), septoria leafspot caused by *Septoria lylopersici* are major diseases (Rebecca *et al*., 2019). Figure 2.1 shows a tomato plant affected by late bright (*Phytopthora infestans*).

Figure 2.1: Early bright lesions on tomato stem and leaflet (Rebbeca *et al*., 2019).

The early signs are manifested on the lower, older leaves as grey–green to yellow spots and color. These diseases in Kenya are treated or prevented using dithiocarbamates fungicides such as mancozeb, mixtures such as mancozeb and metalaxyl or cymoxanil and mancozeb, propineb formulations, copper based fungicides and sulphur (Waiganjo *et al.,* 2006).

2.2 Fungicides

Fungicides are classified in different chemical groups. The main chemical groups are benzimidazoles, dicarboximide, imidazole, piperazine, triazole and phenylamide.A phenylamide such as apron is used to dress some types of seeds sold in the Kenyan market. Strobilurins with sub-classes such as pyraclostrobin (cabrio) and azoxystrobins (quadris) are used in coffee and French beans respectively in Kenya. Cyano-acetamide oximes such as cymoxanil is mixed with mancozeb while chloronitriles (daconil) and sulphurs such as microthiol are other classes of fungicides. Dithiocarbamates is a class considered in this study and includes mancozeb and propineb while others are metiram zineb and nabam.

The mode of action of fungicides can be contact or translaminar. Contact fungicides only protect the plant without uptake. Translaminar (systemic) fungicides are taken and redistributed through xylem to the upper parts of plant.

2.3 Dithiocarbamates (DTCs)

Dithiocarbamates are a group of fungicides (organosulphur compounds) that exist as strong complexes with various metal ions in a polymeric form. They are applied in agriculture as pesticide and in the rubber industry as vulcanization accelerators and antioxidants. They include ethylenebisdithiocarbamates (EBDCs) such as mancozeb, zineb, metiram and propylenebisdithiocarbamates (PBDCs) like propineb. Mancozeb (zinc-manganeese ethylene bisdithiocarbamate) and propineb (zincpropylene-1,2-bisdithiocarbamate) are some of the dithiocarbamate pesticides used as fungicides in agriculture and are considered in this study. Their structural formulae are shown in the figures 2.2 and 2.3.

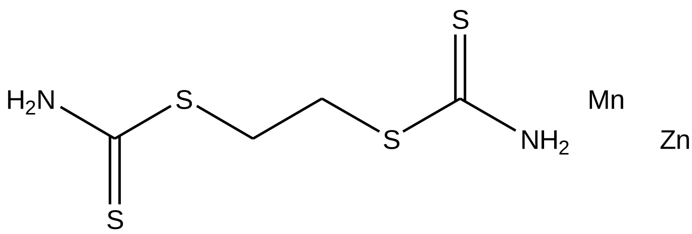

Figure 2.2: Structural formula of mancozeb (Source: EPA.,2010)

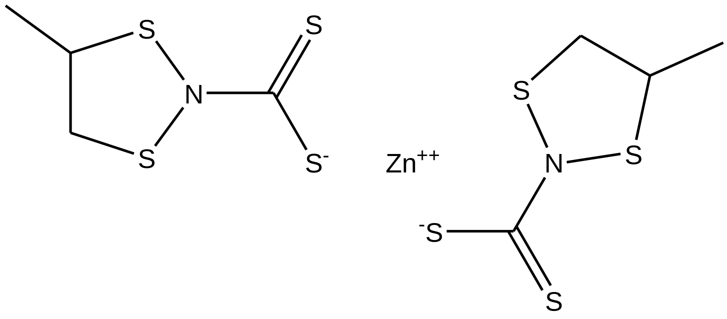

Figure 2.3: Structural formula of propineb (Source :EPA., 2010)

They are preventive fungicides applied before infection and are contact fungicides which act by inhibiting metal dependent and *sulphydryl* enzymes. Mancozeb and propineb or their various mixtures are used in Kenya for spraying on various vegetables such as tomato and sweet pepper as shown on Table1. Dithiocarbamates are known to degrade into different metabolites in the environment, during the cooking process and also during storage (Knio *et al.,* 2000).

2.3.1 Environmental transport and transformation of dithiocarbamates

Dithiocarbamates such as mancozeb and propineb are high molecular weight polymer containing transition metal ions such as zinc, manganese, copper and iron. They are nearly insoluble in water, do no volatilize from water and do not bio-concentrate in the environment. Ethylenebisdithiocarbamates (EBDCs) are unstable in the environment in the presence of moisture, oxygen or in biological systems either basic or alkaline (Gul *et al.*, 2008).

Ethylenebisdithiocarbates decompose forming many bi-products with the elimination of small molecules like hydrogen sulphide and carbon (IV) sulphide. The summarized decomposition pathway is shown in the flow diagram indicated on figure 2.4. The initial stage of decomposition involves the molecule changing to β-amino ethylenedithiocarbamate which further degrades to ethylenethiourea. Ethylenebisdithiocarbamate also decompose to ethylenediisothiocyanate which undergoes hydrolysis to ethylenethiourea (ETU) with IUPAC name 1, 3- ethylene-2-thiourea) with other names such as 2-imidazolinethione and also 4, 5-dihydroimidazole-(3H)- thione.

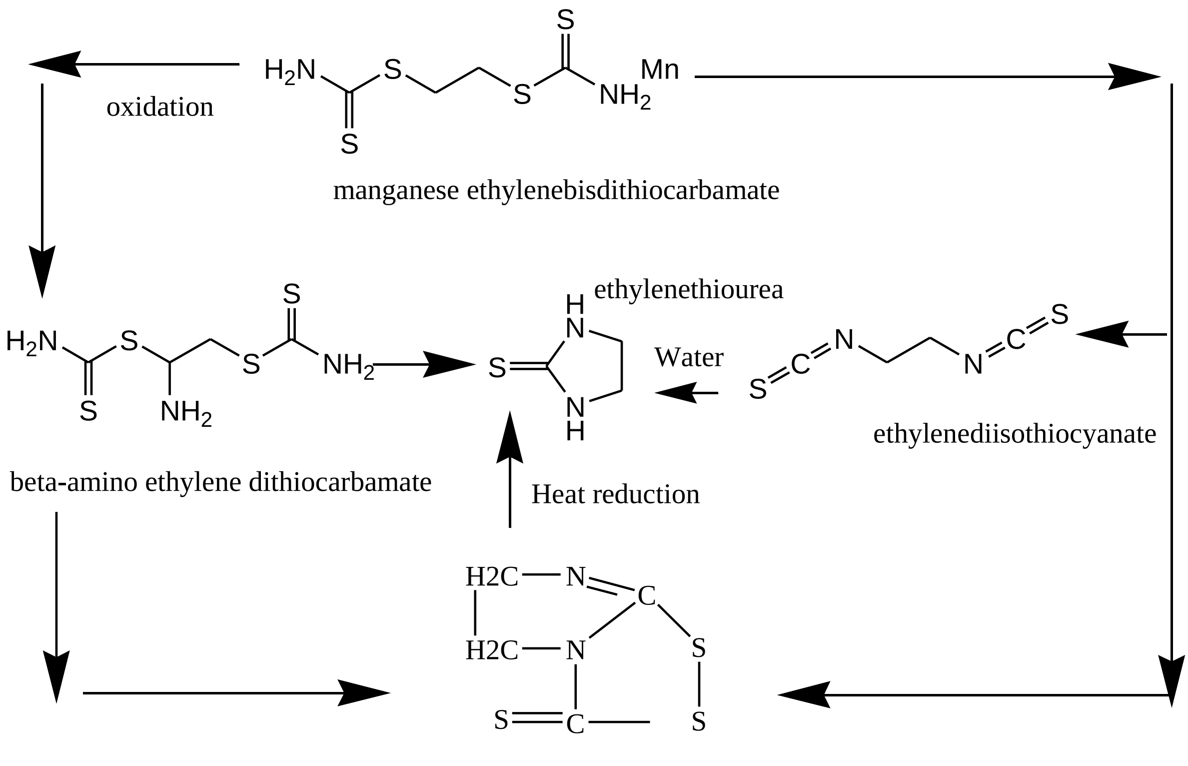

Figure 2.4: Decomposition pathways of EBDC (EPA. 2010).

The metabolite ethylenethiourea (ETU) leaches easily and is water soluble. Propineb degrades to propylenethiourea (PTU) which is not considered in this study.

2.3.2 Dithiocarbamate and ethylenethiourea residues in vegetables

Quality of food is of great concern hence it is important to know the pesticide residues in foods regularly so as to evaluate the risk to consumers for appropriate mitigation. Dithiocarbamate fungicides and the metabolite ethylenethiourea (ETU) residues have been detected at varying concentrations in different parts of the world. ETU residues ranging from 0.08 to 0.11 $mgkg^{-1}$ was detected in two tomato juices obtained from different markets in Istanbul Turkey (Gul and Bucket., 2008). However,residue levels of ethylenethiourea (ETU) when studied and considered alone can lead to some misleading conclusions because the source in foods is due to use of fungicides. ETU is a metabolite of ethylenebisdithiocarbamate fungicides which degrades to this harmful metabolite in the environment and also during storage and cooking. The study on dithiocarbamate residues has been carried out in various parts of the world on different vegetables but any recent study in Kenya lacks in literature search, though Kenya is a large consumer of such fungicides and also an exporter and consumer of horticultural products.

Residue levels of different dithiocarbamates (DTCs) were found above the local legal limit in vegetables obtained from markets in Spain. Peppers showed 96.9% positive results with 15.6% of samples having residues above the European union maximum residue level (MRL) while tomato samples studied showed 87.5% positive results though no sample had values exceeding the set MRL (Lopez *et al.*, 2012). A study done on residue level of dithiocarbamates in field tomato and green house tomatoes in Iran found residues in 81.2% of the samples analyzed with samples exceeding the MRL set by European union and world health organization of 3 mg/kg (Jafari *et al.*, 2012). In Brazil a study carried out showed residue levels ranging from 0.202 to 0.220 mg/kg in tomato (Caldas *et al.*, 2004). In Bukavu district of Congo residue levels of mancozeb ranged from below detection to 4.65 mg/kg showing that 24 % of tomato had levels above European Union MRLs (Mpiana *et al.*, 2014).

Vegetables like tomato and peppers are consumed when raw and cooked accompanied with some process such as simple cleaning. It is imperative to investigate residue levels of dithiocarbamate and the metabolites when such conditions or parameters are considered. Cleaning vegetables with chlorine water has shown a reduction in ethylenebisdithiocarbamate residue levels efficiently (Hwang *et al.*, 2002), while thermal treatment of dithiocabamate maneb showed conversion to ethylenethiourea (ETU) by 28%. Thermal treatment above 100 ºC led to a conversion of the fungicide to ETU by 32% (Kontou *et al*., 2004).

Maximum residue levels (MRL) for different crops and fungicides have been set by different stake holders. The MRL for dithiocarbamates has been set as 3 mg/kg and an acceptable daily intake (ADI) of 0.03mg/kg bw/day while the MRL of ETU has been set as 0.05 mg/kg for tomato and an acceptable daily intake (ADI) of 0.004 mg/kg bw/day (FAO/WHO JMPR., 2004).

2.4 Toxicological effects of dithiocarbamates and ethylenethiourea

The toxicity of dithiocarbamate is considered on its own and also in relation to the harmful metabolites such as carbon sulphide and ethylenethiourea. In Germany ETU is classified as a 3B carcinogen in animals while Finland, France, Sweden and the USA also list ethylenethiourea as a carcinogen (FAO/WHO JMPR., 2010). Mancozeb is an example of cholinesterase inhibitors which affects the nervous system in Human and manifests in form of headache, blurred vision and nausea among other symptoms. At high doses convulsions, slurred speech**,** confusion, slowed heartbeat are observed. It can also cause skin irritation/rash in small doses (Lopez *et al.*, 2012). Many studies have shown that mancozeb can enter through placental barrier hence affecting the unborn by increasing tumor development (Shukla *et al*., 2001). Mancozeb mainly affects the thyroid gland which is manifested as alterations in thyroid hormones, increased thyroid weight, microscopic thyroid lesions (mainly thyroid follicular cell hyperplasia) and thyroid tumors. In rats injury to peripheral nerves which is referred to as microscopic neuropathy has been reported. A metabolism study (in vivo) indicates a 7.5% conversion of ethlenebisdithiocarbamates to ethylenethiourea (EPA. 2010).

Mancozeb is not acutely toxic when taken through the oral, dermal or inhalation routes though it causes eye irritation. The acute oral and dermal toxicity is given as LD_{50} of 5000 mg/kg with an acute eye irritation leading to corneal damage in less than 7 days on continuous exposure and also known to cause spontaneous abortions in rabbits on a dose of 80 mg/kg/day (Kroes *et al*., 2004). Carbon sulphide exposure is known to lead to cardiovascular disease and is also neurotoxic. Ethylenethiourea (ETU) which is a metabolite of fungicides such as mancozeb, zineb, metiram and maneb has a half-life of 1-7 days in the open environment and between 5-10 weeks in the soil. ETU can pass through placental barrier and also causes thyroid and carcinogenic effects on test animals (Shukla *et al.,* 2001).ETU which is known to have thyroid effects, liver effects, developmental effects, also known to be teratogenic ,mutagenic and also carcinogenic (Teramoto *et al*., 2005).

The carcinogenicity of ETU and EBDCs cannot be considered in isolation of the other. A study on rats showed thyroid follicular cell adenomas and carcinomas when high doses of mancozeb were given to both male and females. The same study also showed hormonal level rise and increased thyroid weight leading to a classification of mancozeb and ETU as a B2 (probable human carcinogen). ETU has been given a cancer potency factor (Q_1) of 0.0601 mg/kg/day by the Environmental protection agency of the United States of America (EPA. 2010).

Dithiocarbamates in combination with nitrite arising from foliar application of nitrogenous fertilizers with nitrates is also another health concern. Nitrites are formed by reduction in vegetables after cooking, in human saliva or in meats especially when cooked. Dithiocarbamates in the presence of nitrite can be converted to *N*-nitroso which may be a carcinogenic derivative. Such derivatives include *N*-nitrosodimethylamine (NDMA) and nitrosoethylenethiourea (Debbar and Moore. 2002).

2.5 Methods of analysis

Methods of analysis of ethylenethiourea (ETU) and dithiocarbamates (DTCs) separately or simultaneously in fruits, vegetables, foods, beverages, cigarette smoke, body fluids, air and on formulated fungicides include thin layer chromatography, gas chromatography and many others. The dominant methods used for determining DTCs and metabolites are based on their decomposition to carbon (IV) sulphide in acidic medium then analysis by gas chromatography (GC) sometimes when coupled with mass spectrometry (MS). The GC methods are time consuming and also known to give carbon (IV) sulphide blinds arising from plants such as cabbages in the *Brassica* family which have secondary sulphur metabolism. More recent analysis of dithiocarbamates and their metabolites have employed use of high pressure liquid chromatography coupled with a diode array detector (HPLC-DAD). Some studies include determination of maneb and metabolites like ethylenethiourea in tomatoes (Garcinuno *et al.,* 2004) and analysis of fruit juice for ETU (Gul and Buket. 2008).

There are other studies using HPLC-DAD such as the study done in Iran for determining residues of dithiocarbamates; mancozeb , thiram and propineb in tomatoes (Jafari *et al*., 2013) and another study done on in Valencia Spain for residues of propineb, mancozeb and maneb (Lopez *et al.*, 2012) of which both studies analyzed the dithiocarbamatess simultaneously with distinct retention times. Coupling the HPLC with mass spectrometry (MS) has been done in different studies such as HPLC-MS (Blasco *et al*., 2004), HPLC-MS/MS (Crnograc *et al.*, 2008) among others. In this study simultaneous determination of two dithiocarbamates and the metabolite ethylenethiourea was done with HPLC using a diode array detector (DAD).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Research design

This research consisted of two main parts. The first part of the study was the determination of the residue levels of mancozeb, propineb and ethylenethiourea (ETU) in tomato and sweet pepper from markets. This part of the study started from the month of February ending at June (dry and wet seasons) in the year 2014. The second part of the research involved investigation of the effect of cooking temperature and cleaning treatments on the levels of dithiocarbamates and ethylenethiourea using samples grown in a study garden. The study involved planting tomato and sweet pepper, spraying and then harvesting the vegetables for easy monitoring residue levels of ethylenethiourea (ETU) and the dithiocarbamate mancozeb at different cooking temperatures ranging from 25 ℃ (room temperature as T_1) to 100 ℃ as (T_6) and different cleaning treatments

3.2 Area of study

The study areas were Kirinyaga and Nairobi Counties, with the former located on the slopes of Mount Kenya south of Equator and a climate that is appropriate for growing different vegetables. The latter is the capital city with a large consumer population and also the home to head offices of various international organizations.

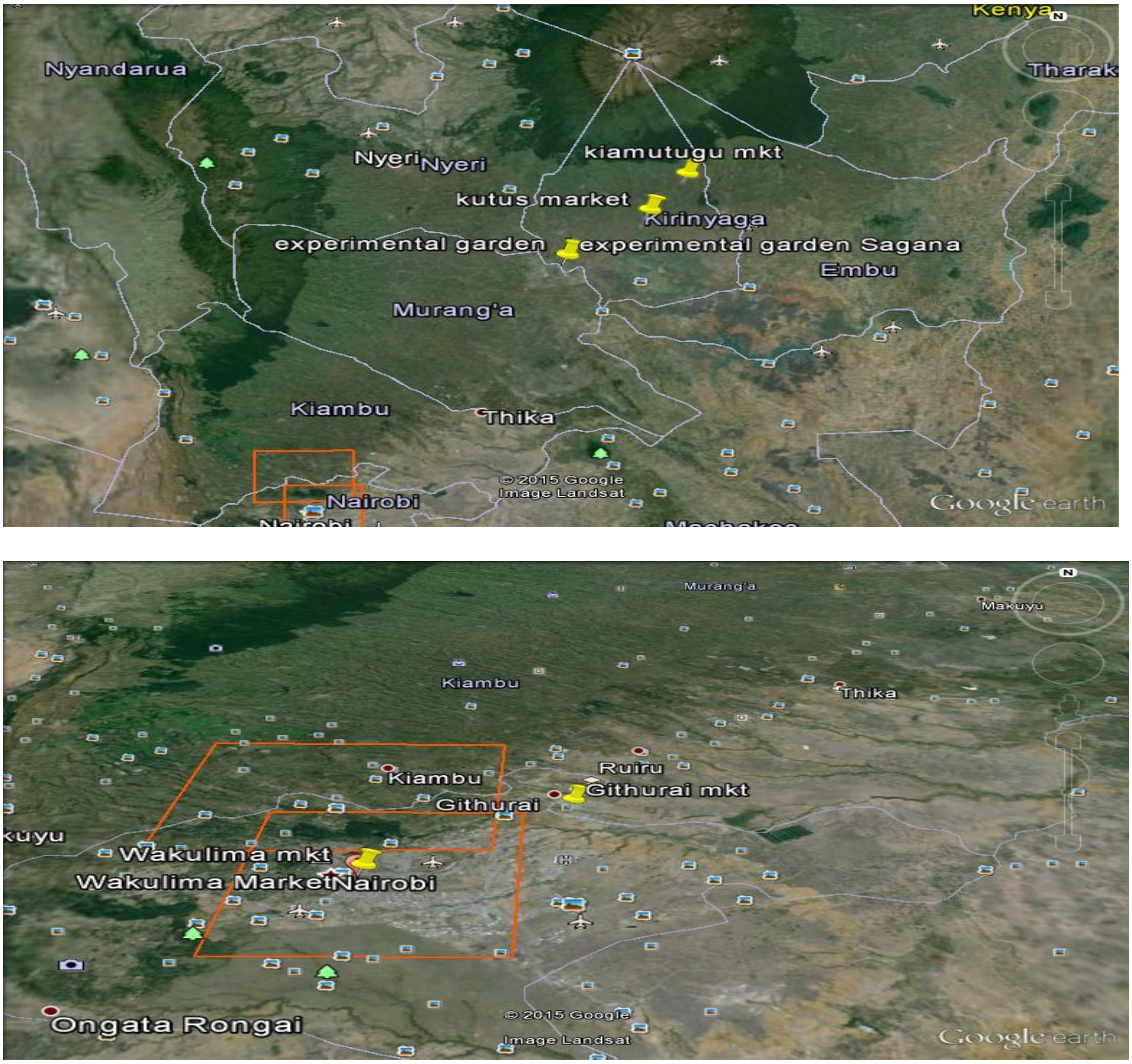

Figure 3.1: Map showing the study area in Kirinyaga County and Nairobi County

(Source: Google earth)

The sampling sites which were Kiamutugu and Kutus markets together the study garden Sagana are shown on Figure 3.1 with the yellow place marks within the latitudes 0^{o} $35^{'}42$, 91” S to 0^{o} $28^{'}3$, 15” S and longitude 37° $12^{'}$ 30, 33^{0} E to 37° $23^{'}21$,84^{0} E. Some samples were obtained from Githurai and Wakulima markets in Nairobi County the capital city of Kenya with a metropolitan population of about 5 million. The markets are within latitudes 1^{o} $13^{'}4$, 65" S to 1^{o} $17^{'}15$, 21" S and longitudes 36° $55^{'}13,18^{0}$ E to 36° $49^{'}55,99^{0}$ E respectively.

3.3 The study garden

The land for the study garden was initially ploughed using an oxen plough. Seed beds were prepared on the same land by breaking soil into fine particles. Two varieties of certified seeds, Rambo F1 (Lot no. 13-5182, batch number INML05013038) and Kilele F1 (Lot no, 13-5871-3, batch number 10656342) and sweet pepper variety, California Wonder (obtained from Griffaton France), were grown on seed beds. The seedlings were sprayed with fungicides, insecticides and foliar on germination. The tomato seedlings matured after 30 days while sweet pepper matured after 35 days.

The seedlings were transplanted in three separate plots measuring 2 × 2 meters for each type of vegetable in ploughed trenches. The trenches were 15 cm deep with a spacing of 75 cm between trenches and 45 cm between plants in a row. Organic manure (cattle and goat manure) and commercial fertilizer NPK 17.17.17) were applied then the soil in the trenches was homogenized, watered before planting and regularly during growth. Overhead spraying was done using mancozeb and propineb on two separate plots for each vegetable according to manufacturer's instructions of 2.5 grams of fungicide per liter of water. This was done for each vegetable type with intervals of 10 days, up to and including maturity. The gardens (Figure 3.2), were separated by polythene barriers to prevent drifting of fungicides during spraying.

The third plot for each vegetable was sprayed with copper hydroxide based fungicides (formulations which had no DTCs) hence acting as the control. Samples were collected on maturity 75 days after transplanting for analyzing the effect of cooking.

Figure 3.2: Sweet pepper and tomato at the study garden (Source: self)

3.4 Sampling and sample pretreatment

Tomato and sweet pepper samples were obtained in quantities of one kilogram from six different sellers per market. The vegetables were pooled together and then a one kilogram sample taken as a representative sample for the market. Three samples of each vegetable were studied from each of the four markets, Githurai and Wakulima (Nairobi County) and Kiamutugu and Kutus (Kirinyaga County during dry and wet seasons (Figure 3.3). Samples were collected in the morning hours then packed in polythene bags, transported the same day in a cold box and stored in a refrigerator (deep freezer) awaiting extraction and high pressure liquid chromatography analysis.

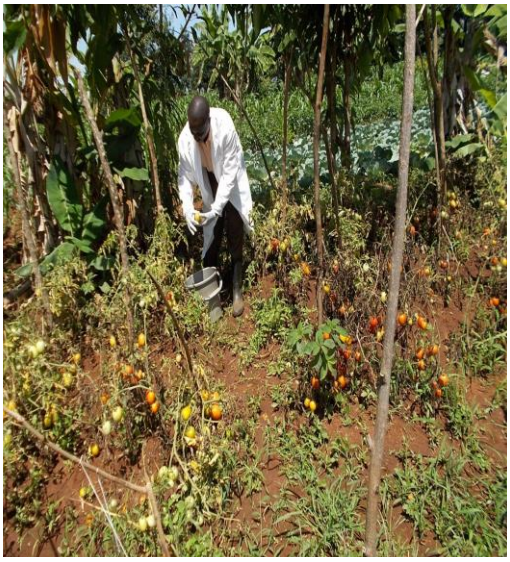 

Figure 3.3: Sample collection from the garden and market (source: self)

Samples for temperature treatment and washing treatment were collected from the experimental gardens sprayed with mancozeb and propineb by purposive sampling from all the plants one day after spraying (Figure 3.3). The samples were collected separately according to vegetable type and fungicide sprayed then pooled together. A one kilogram quantity was used as the representative sample for each vegetable per fungicide. The samples were packed in plastic bags in a cold box then transported same day and stored in a deep freezer.

3.5 Cleaning of containers

Plastic apparatus were not used in the extraction due to the nature of solvents used. Glass ware was soaked for 10 hours using fresh hydrochloric acid, soaked in distilled and de-ionized water for 8 hours, rinsed with fresh distilled and de-ionized water then dried on a rack in an oven at temperatures of 100 ℃**.**

3.6 Chemicals and reagents

The standards ethylenethiourea (98% purity), mancozeb (96.8% purity) and propineb (103% purity) analytical grade were sourced from a company Pestanal through Kobian Kenya limited. The HPLC solvents (acetonitrile, chloroform, dichloromethane, distilled water, methanol) and sodium dodecyl sulphate (SDS) were analytical grade obtained from Kobian Kenya limited while nitrogen gas was obtained from British Oxygen Company Kenya limited.

3.7 Instrumentation

The HPLC instrument (Shimadzu model LC-20A) was used. The machine had a Quaternary pump (LC-20AD), PDA detector (SPD-M20A), system controller (CBM-20A), auto sampler (SIL-20A) and column oven (CTO-10ASVP). The machine was connected to a HP computer with a software Shimadzu LC solution (Figure 3.4). The HPLC column used was EC 250/4.6 NUCLEODUR 100-5 C18ec Batch 34500033 (C-18 5µm particles and length 250 mm×4.6 mm internal diameter) obtained from Macherey Nagel Germany and supplied by Chemo quip Kenya limited. The mobile phase was a mixture of acetonitrile - methanol-100 mM sodium dodecyl sulfate (SDS).

Figure 3.4: The HPLC machine (source**:** self)

The amber colored vials of 0.5 ml containing the samples and external standards were placed in the machine and analyzed with an injection volume of 20 µl and each sample was run in triplicate. The column separation conditions were such that; the 95% sodium dodecyl sulphate (SDS) + 5% acetonitrile connected to Pump A started at 100% for 2 minutes while the pumps B, C and D were at 0% . At 2.01 minutes Pumps B, C and D were run at 30% 0.1M SDS, 33% methanol and 37% ACN up to 6.00 minutes while pump A was at 0%. At 6.01 minutes Pump A was run at 100% up to 10.00 minutes while the other three pumps were at 0%. The detector end time was 10 minutes while the LC stop time was 15 minutes. The elution program has similarities with another study done on tomato for Maneb and ETU as in Garcinuno *et al.*, (2004) and is shown in Appendix XII. Other conditions were a flow rate of 0.9 ml/min and an oven temperature of 40 ℃ . Quantification was by multi-wavelength monitoring done with a band width of 4 nm and absorbance spectra recorded from 200-400 nm.

3.8 Laboratory procedures

The laboratory procedures used in the study are discussed in this section. They include standards preparation, method validation, extraction, simulated cooking and the procedures used for chemical cleaning.

3.8.1 Preparation of standards

The stock solutions of standards with a concentration of 1000 mg/kg of propineb and mancozeb were prepared by dissolving 10 mg in10 ml acetonitrile (Appendix VI). The stock solution of ETU with a concentration of 250 mg/kg was made by dissolving 12.5 mg in 50 ml of acetonitrile and stored in a freezer.

3.8.2 Method validation

Recovery was determined using unsprayed vegetables which had no dithiocarbamate fungicides, obtained from the study garden. A quantity of 1g of each vegetable sample were spiked by use of 500 µl of a solution with a concentration of 100 mg/kg of each analyte then homogenized by shaking. The spiked samples were maintained for 30 minutes for penetration of the standards before extraction. The data and calculations are shown in the results section.

Calibration was done by preparing a series of dilutions containing each external standard (mancozeb, propineb and ethylenethiourea) from 0.1 to 100 mg/kg. The calibration curve of ethylenethiourea was run at 10, 20, 50, 60, 80 and 100 mg/kg while the curves of propineb and mancozeb were both ran at 2.5, 5, 10, 15, 20 and 25 mg/kg. Calibration curves were obtained by plotting peak area against concentrations of standards between the given ranges. Limit of blank (LOB) was determined by running the extractant of six blank samples without the analyte of interest and their standard deviations while the limit of detection (LOD) was determined by determining the standard deviation of six low concentration samples for each analyte and adding to LOD using appropriate equations given in the results of this study (David and Terry., 2008).

3.8.3 Extraction procedure

The vegetables were finely chopped separately using a knife. A 1.0 gram sub-sample was placed in a 50 ml beaker containing a volume of 3 ml of a mixture of acetonitrile, dichloromethane and chloroform in the ratio of 1:1:1. Extraction was achieved by 2 minutes of mechanical shaking. The suspension obtained was filtered through a filter paper in a Buchner funnel and rinsed with 2 ml of extractant followed by addition of 2 ml of methanol to filtrate. The mixture was then evaporated to dryness under a gentle stream of nitrogen gas at room temperature. Residues obtained were dissolved in 500 µl of a solution of acetonitrile and water in the ratio of 1:1. The solution was filtered through a Millipore 0.45 µm nylon syringe into sample vials then stored in a freezer before HPLC analysis (Garcinuno *et al*., 2004). The extraction was done in triplicate.

3.8.4 Simulated cooking

The experiment was done using three different samples for each vegetable. A one kilogram sample of tomato obtained from the plot sprayed with mancozeb was cut into small pieces with a knife and homogenized, then divided into five equal portions of 200 g. Each of the 200 g portions was divided into two, 100 g portions. From the first portion 3 small portions weighing 1.0 g of the uncooked tomato were extracted and analyzed while the second portion of 100 g were placed in a 250 ml round bottomed flask and connected to a vertical condenser. Heating using a mantle was done at 60 ℃ for 10 minutes followed by cooling (Appendix I). On cooling extraction was done on one gram sub samples in triplicate as shown in the extraction procedure. The procedure above was repeated at different temperatures with intervals of 10 ℃ up to 100 ℃ for tomato and sweet pepper. Solutions obtained were stored in amber colored vials in a deep freezer awaiting analysis. The procedure in this section was repeated two more times with the other samples obtained for both vegetables.

3.8.5 Cleaning procedure

The study on the effect of different types of cleaning methods of sweet pepper and tomatoes on residue levels involved use pre-sprayed vegetables. The samples of three kilograms for each vegetable were collected from the study gardens sprayed separately with mancozeb and propineb. One sample was divided into three equal portions (1 kg each) for cleaning with distilled water, sodium hypochlorite solution and one unclean. The first portion of tomato sprayed with propineb was cleaned by placing sample in a bowl containing five liters of distilled water for four minutes and rinsed twice. The sample was allowed to dry for one hour then cut into small pieces and homogenized. From the homogenized tomato six different one gram sub samples were obtained and extracted according to the extraction procedure. The procedure was repeated with the second portion of tomato which was cleaned with five liters of a 1% sodium hypochlorite solution for 4 minutes and rinsed twice with two liters of distilled water. A third portion of tomato which was unclean was processed according to the extraction procedure. The above procedures were repeated using sweet pepper samples obtained from the study garden sprayed with propineb and also using tomato and sweet pepper samples obtained from the study gardens sprayed with mancozeb. The extracted samples for tomato and sweet pepper were analyzed for propineb, mancozeb and ethylenethiourea. The procedure in this section was done six times for separate samples of each vegetable.

3.9 Data analysis

The data was analyzed using analysis of variance (ANOVA) One way to determine significant differences. The differences include residue levels in the vegetables in different seasons, different markets, different cooking temperatures and two different washing methods. The mean values obtained were compared with the maximum residue levels (MRLs) set by the WHO, EPA and EU.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Introduction

The results for the validation of the methods of analysis used and discussion of the results for the study are presented in the sections that follow.

4.2 Method validation

The methods used in this study were validated by use of calibration curves for the different standards where regression equations, correlation coefficients and limits of detection were obtained. Recovery data, limit of blank and chromatogram separation data are also provided.

4.2.1 Calibration

The calibration curves for, ethylenethiourea, mancozeb and propineb were obtained. The curves are shown in Appendices II and III and IV respectively. The regression equations and correlation coefficients for mancozeb, ETU and propineb together with the limits of detection (LOD) and limits of blank (LOB) are shown in Table 4.1.

Table 4.1: Calibration data

Analyte	Reg. equation	R^2	LOD	LOB
Ethylenethiourea	Y=21706X+9159	0.999	0.0100	0.0085
Mancozeb	Y=2971X+5187	0.948	0.0990	0.0080
Propineb	Y=2171.7X+297.79	0.996	0.6500	0.0900

The R^2 values (0.948-0.999) make the study appropriate for use in analysis of residue levels of dithiocarbamate fungicides and the metabolite. The R^2 values indicate that the established calibration curves are linear over the respective range of concentrations, explaining that more than 94.8% correlation between concentration and responses. The correlation coefficients relates with other studies on same compounds such as Garcinuno *et al*. (2004), Gul and Buket (2008) and Jafari *et al*. (2010). The limits of detection (LOD) and limits of blank (LOB) values show the method is sensitive enough hence can measure small concentrations of analytes in the samples and clearly differentiate from noise. They were calculated according to Equation (1) and (2) (David and Terry. 2008).

$$\text{LOB} = \text{mean Blank} + 1.645\ (\text{SD blank}) \qquad \text{Equation 1}$$

$$\text{LOD} = \text{LOB} + 1.645\ (\text{SD low concentration sample}) \qquad \text{Equation 2}$$

The limits of blank range from 0.09 mg/kg for propineb, 0.0085 mg/kg for ethylenethiourea (ETU) and 0.008 mg/kg for mancozeb,while the limits of detection ranged from 0.01 mg/kg for ETU to 0.099 mg/kg for mancozeb. Blasco *et* al. (2004) obtained the limit of detection (LOD) of 0.01 mg/kg for ETU and 0.1 mg/kg for maneb. In another study done in Iran, the LOD of 0.1 mg/kg for mancozeb and propineb was attained (Jafari *et* al., 2012). The values show that the method is robust for the analysis of the fungicides and the metabolite in vegetables studied, since results are consistent with other studies.

4.2.2 Recovery studies

Recovery studies were evaluated by recovery test, and are shown in Table 4.2

Table 4.2: Accuracy by recovery test

Parameter	C_U (mg/kg)	C_A (mg/kg)	C_F (mg/kg)	%Recovery
Mancozeb	91.26	100	191.26	91.26
ETU	95.89	100	195.89	95.89
Propineb	95.26	100	195.26	95.26

The recovery was calculated according to Equation 3 (David and Terry, 2008);

$$\% \text{ Recovery } = \frac{C_F - C_U}{C_A} \text{ x } 100 \qquad \text{Equation 3}$$

Where C_U represents the concentration in unfortified sample; C_A is the concentration of fortification (spiking solution), C_F is the concentration determined in fortified sample after extraction.. The percentage recovery lies within the range 91.26% to 95.26%. The recovery studies compare favourably with other studies where 94.15% recovery for Maneb and 96.46% for ETU was achieved (Gul and Buket, 2008) which confirms the method fit for analysis of the substances done in this study.

4.2.3 Chromatographic methods

The high pressure liquid chromatographic (HPLC) analysis of the three analytes of interest was done using a simultaneous analysis and peak identification. The chromatograms for one of the standards ethylenethiourea and one of the samples from one of the markets studied which had all the analytes detected are shown in appendix

VII and VIII and discussed in this section. The others which show the chromatograms of mancozeb, propineb standards and a sample of sweet pepper are shown in appendices IX, X and XI respectively. The chromatograms show the retention time of the various peaks together with tables showing the peak area. The retention times in the chromatograms of standard and sample were 3.2±0.2 minutes for ethylenethiourea (ETU) ,while that of propineb was 3.9 ±0.2 minutes and that of mancozeb was 8.7±0.2. The retention times achieved in this study are similar to other studies such as Gul and Buket, (2008) where; using a C18 column the retention time of ETU was 3.3 minutes that of maneb was 4.1 minutes while that of mancozeb was 9.1 minutes. Quantification was done by multiplying the amount obtained from the graph (using the regression equations for standard) with a dilution factor of 0.5 as shown in Equation 4;

$$Q\left(\frac{mg}{kg}\right) = \left(\frac{P - C}{M}\right) \times 0.5 \qquad \text{Equation 4}$$

Where Q (X in equation) is the levels in mg/kg, P is the peak area (Y) , C is the Y intercept while M is the gradient of the regression equation.

4.3 Residue levels in vegetables during dry and wet seasons

Tomato and sweet pepper samples from different markets during wet and dry seasons were analyzed for propineb, mancozeb and ETU. The results are discussed in the following sections.

4.3.1 Residue levels of propineb

The results showing mean residue levels of propineb are presented on Table 4.3. The propineb residues in tomato ranged from 0.83±0.05 to 3.97±0.50 mg/kg during the wet season with tomato samples from Kutus market having the highest level and Githurai market having the least. During the dry season the tomatoes from three markets had values below detection limit (BDL), however tomatoes from one market (Githurai) had propineb mean residues of 1.91±0.08 mg/kg during the wet season, tomatoes from different markets showed significant difference in the residue levels of propineb on tomato. The results also show that tomato samples obtained during the dry season gave the highest number of results below detection limit. This can be explained from common agricultural practices, whereby frequency of spray is increased in wet season but decreased in dry season (Mugambi, 2009). The propineb residue on sweet pepper ranged from BDL to 6.54±0.72 mg/kg during the wet season with Kiamutugu market pepper samples having the highest levels at a mean of 6.54±0.72 mg/kg. During the dry season propineb residues on sweet pepper ranged from BDL to 2.91±0.06 mg/kg with the sweet pepper samples from Githurai market having the highest level.

Table 4.3: Mean levels of propineb in vegetables during dry and wet seasons

	tomato (mg/kg) n=3		sweet pepper (mg/kg) n=3	
Market	**wet**	**dry**	**wet**	**dry**
Wakulima	3.15 ± 0.08^{Cd}	BDL	BDL	BDL
Githurai	0.83 ± 0.05^{Ab}	1.91 ± 0.08^{C}	BDL	2.91 ± 0.06^{Ac}
Kutus	3.97 ± 0.50^{dE}	BDL	0.99 ± 0.13^{aB}	0.87 ± 0.04^{Bb}
Kiamutugu	1.48 ± 0.87^{Bc}	BDL	6.54 ± 0.72^{bD}	1.09^{cC}
P-values	< 0.001	< 0.001	< 0.001	< 0.001
LSD	0.04	1.00	0.03	1.00

Mean values followed by the same small letter within the same column do not differ significantly from one another while those followed by the same capital letter in the same row do not differ significantly.

From Table 4.3 the mean residue levels of propineb on the sweet pepper vegetable differed significantly on comparing Kutus market and Kiamutugu market during the wet and dry seasons and also Githurai and Kutus during dry season but did not differ significantly when the dry and wet seasons were compared in Kutus market. The lack of any significant difference can be explained by low usage of the fungicide (Waiganjo *et al*., 2006).

The levels of propineb on tomato samples from Wakulima and Kutus markets (3.15±0.08 and 3.97±0.5 mg/kg, respectively) and sweet pepper samples from Kiamutugu market (6.54±0.72 mg/kg), were above the maximum residue levels (MRL) set by FAO/WHO of 3mg/kg (FAO/WHO., 2004). The results on residue levels of propineb from this study correlates with similar studies such as that of green houses and non green houses tomatoes in Iran, which found residues in one green house tomato sample above the set MRL at 3.2 mg/kg (Jafari *et al*., 2012). Another study in Bukavu District of Congo had tomato samples with residues of mancozeb at 4.65 mg/kg which is above the MRL of European Union and FAO/WHO (Mpiana *et al*., 2014).

4.3.2. Residue levels of mancozeb

The mean residue levels of the fungicide mancozeb in tomato and sweet pepper obtained from markets during different seasons are shown on Table 4.4.

Table 4.4: Mean levels of mancozeb in vegetables during wet and dry seasons

	tomato (mg/kg) n=3		sweet pepper (mg/kg) n=3	
Market	**wet**	**dry**	**wet**	**dry**
Wakulima	BDL	0.25 ± 0.09^{aA}	BDL	0.16 ± 0.00^{Aa}
Githurai	1.29 ± 0.04^{aB}	1.06 ± 0.07^{bC}	BDL	2.21 ± 0.86^{Bc}
Kutus	2.10 ± 0.03^{bD}	1.60 ± 0.19^{cB}	1.49 ± 0.08^{aB}	2.52 ± 0.77^{bC}
Kiamutugu	2.56 ± 0.12^{c}	BDL	2.69 ± 0.57^{bC}	2.13 ± 0.27^{Bc}
P-values	< 0.001	< 0.001	< 0.001	0.004
LSD	0.01	0.02	0.02	0.03

Mean values followed by the same small letter within the same column do not differ significantly from one another while mean values followed by the same capital letter within same row do not differ significantly

From Table 4.4, the mean residues of mancozeb in tomato ranged from below detection limit (BDL) to a mean of 2.56±0.12 mg/kg during the wet season and from 0.25±0.09 to 1.60± 0.19 mg/kg during dry season. The residues during the wet season were significantly higher than those obtained during the dry season in Kutus market while in Kiamutugu market the wet season had high residues at 2.56±0.12 mg/kg compared to BDL in the dry season. Within seasons different markets showed significant differences of mancozeb residue levels on tomato. The mean levels of mancozeb on sweet pepper ranged from BDL in two markets to a mean of 2.69±0.57 mg/kg during the wet season. The sweet pepper samples obtained during the dry season had mancozeb residue values ranging from 0.16±0.00 to 2.52±0.77 mg/kg. Different markets had significant difference in the levels of mancozeb on sweet pepper within a season. The residues were significantly higher during the dry season than during the wet season in Kutus market and other markets with the exception of Kiamutugu. The significance can be attributed to wash off during wet season.

The results from the study indicated there were no mancozeb residues in tomato and sweet pepper which were above the MRL set by FAO/WHO of 3mg/kg. This may be attributed to cleaning or polishing to remove surface dirt in the markets before selling a limitation of this study and also the high decomposition rate of mancozeb in the environment (Olaniyi *et al*., 2010). The study findings compare favorably with those obtained from other studies, such as Spain where no residues of mancozeb above MRL were noted in tomato vegetable (Lopez *et al*., 2012). Another study done in commercial fruit juices obtained from Instabul found no residues above the MRL (Gul and Buket, 2008). Other studies which had similar findings were done in Iran Jafari *et al*. (2012), Turkey Mehmet *et al.* (2014) and Brazil Caldas *et al*. (2004).

4.3.3. Mean levels of ethylenethiourea

The mean residue levels of the metabolite ethylenethiourea in both vegetables obtained from various markets are shown on Table 4.5.

Table 4.5: Mean levels of ethylenethiourea in vegetables during different seasons

	tomato (mg/kg) n=3		**sweet pepper (mg/kg) n=3**	
Market	**wet season**	**dry season**	**wet season**	**dry season**
Wakulima	BDL	21.99±5.19^{bC}	BDL	8.88±1.55^{bD}
Githurai	8.52±1.25^{bB}	9.30±1.82^{aB}	BDL	0.65±0.16^{aA}
Kutus	9.36±0.78^{bB}	27.99±0.67^{bD}	0.56±0.24^{aB}	1.63±0.03^{bC}
Kiamutugu	1.81±0.21^{aA}	BDL	7.23±1.24^{bC}	1.53±0.04^{bD}
P-values	$<$ 0.001	0.001	$<$ 0.001	$<$ 0.001
LSD	0.02	0.03	0.39	0.16

Mean values followed by the same small letter within the same column do not differ significantly from one another while mean values followed by the same capital letter within same row do not differ significantly

From Table 4.5 the residue levels of ethylenethiourea (ETU) in vegetables ranged from below detection limit (BDL) to 27.99±0.67 mg/kg. The tomato samples had values ranging from below detection limit (BDL) to 9.36 ±0.78 mg/kg in the wet season and

BDL to 27.99±0.67 mg/kg in the dry season with tomato from Kiamutugu having lowest positive value at 1.81±0.21mg/kg while Kutus had the highest mean at 9.36±0.78 mg/kg in the wet season. In the dry season the lowest positive value was at 9.30 ±1.82 mg/kg from samples obtained at Githurai market. The residue levels of ethylenethiourea (ETU) in tomato obtained during the dry season were significantly higher than those found during the wet season in Kutus market and Wakulima market with no significant difference in Githurai. The high mean residues in dry season is attributed to environmental decomposition of dithiocarbamates and also accumulation of ETU from spraying due to the ETU long lifetime in the environment among other reasons, while the low levels during wet season is attributable mainly to the high water solubility of ETU at 1000 mg/l at 20 ºC (Pallavi and Ajay., 2013). Mean residue levels from different markets differed significantly during dry season while during the wet season, only Kiamutugu and Kutus markets did differ significantly.

Sweet pepper had ETU residues above detection limit in only two markets from Kirinyaga County during the wet season with quantities of 0.56±0.24 mg/kg at Kutus market and 7.23±1.24 mg/kg at Kiamutugu market which differed significantly. During the dry season the mean residues of ETU in sweet pepper were 0.65±0.16 mg/kg from Githurai market to 8.88±1.55 mg/kg in Wakulima market. The residues from Wakulima market differed significantly from the ones from three markets while those from other markets had values which did not differ significantly from each other. The residues of ETU on sweet pepper did differ significantly between the different seasons in Kiamutugu market and Wakulima while in Kutus no significant difference. The results correlate with studies such as Gul and Buket (2008) where residue levels of ETU were noted in two tomato juices samples obtained from Instabul Turkey at 0.08 and 0.11 mg/kg respectively and also from Kenya, Kariuki (2008) where levels of ETU were 10.3 ± 0.6 to 17.5 ± 0.1mg/kg. The mean residues in the vegetables were above the maximum residue limit (MRL) of 0.5 mg/kg set by the Food and Agricultural Organization (FAO) and the World Health Organization (WHO) (FAO/WHO., 2013).

4.4 Effect of cooking on the levels of mancozeb and ETU in tomato and sweet pepper

The results discussed in this section are shown on Table 4.6. On heating from room temperature (25 ℃) the residue levels in tomato increased from 9.43±0.03 to 12.43±0.38 mg/kg at 90 ℃ and then decreased to 10.60±0.03 mg/kg at 100 ℃ showing a conversion of 57.36 % of the initial mancozeb. The ETU residues decrease (Appendix V) at 100 ℃ due to decomposition of ETU at that temperature)

Table 4.6: Effect of temperature on ETU and mancozeb residue in vegetables

temperature°C	tomato (mg/kg) n=3		sweet pepper (mg/kg) n=3	
	etu	mancozeb	etu	mancozeb
25 (room temp)	9.43±0.03[a]	5.23±0.03	2.26±0.01[a]	4.13±0.14
60	10.48±0.15[b]	BDL	2.40±0.05[b]	BDL
70	11.57±0.09[c]	BDL	2.83±0.03[c]	BDL
80	11.91±0.01[d]	BDL	5.09±0.01[e]	BDL
90	12.43±0.38[e]	BDL	5.29±0.04[f]	BDL
100	10.60±0.03[b]	BDL	3.08±0.01[d]	BDL
P- Value	0.003		0.003	

Mean values followed by the same small letter within the same column do not differ significantly from one another

On the sweet pepper vegetable the ETU residues rose steadily from 2.26±0.01 to 5.29±0.04 mg/kg at 90 ℃ **.** When the temperature was increased to 100 ℃ the levels of ETU decreased to 3.08±0.01 mg/kg. The results indicated a complete decomposition of mancozeb to levels below detection limit when the vegetables were heated from room temperature to 60 ℃ with levels decreasing from 4.13±0.1 and 5.23±0.03 mg/kg in sweet pepper and tomato, respectively. The results obtained from the study shows that cooking vegetables containing mancozeb reduces the levels while the levels of ETU increases. The ammounts of mancozeb and ethylenethiourea cannot add up using this method of analysis since the initial mancozeb decomposes to other metabolites like carbon disulphide and ethylene urea which were not quantified in this study.

The results from this study compare favourably with other studies done such as Kontou *et al* (2004) where it was found that there was no decline in ethylenethiourea recovery even on heating at high temperatures such as 90 ℃ even for upto 80 minutes however a conversion of dithiocarbamates to ethylenethiourea was as high as 32±1%, results also compare with other studies which notes that mancozeb like other ethylenebisdithiocarbamates decomposes at cooking temperatures to very low levels while ETU levels rises (Knio *et* al., 2000).

4.5 Effect of cleaning on residue level

The results of the effect of cleaning method on levels of propineb, mancozeb and ETU in tomatoes and sweet pepper are discussed in the sections that follow. The results are used to show the variation of residues on using water only and combining water and sodium hypochlorite (chlorine) when compared with unclean.

4.5.1 Effect of cleaning method on residue levels of propineb in vegetables

The results showing mean residue levels of propineb are presented on Table 4.7.

Table 4.7: Effect of cleaning method on residue levels of propineb on vegetables

	tomato		**sweet pepper**	
cleaning treatment	**mg/kg : n=6**	**% reduction**	**mg/kg: n=6**	**% reduction**
Uncleaned	9.26 ± 0.09^{c}	0.0	10.54 ± 0.20^{b}	0.0
Water only	5.02 ± 0.19^{b}	45.8	9.99 ± 0.18^{b}	5.21
Chlorine	1.83 ± 0.04^{a}	65.1	3.69 ± 0.23^{a}	65.1
p-value	<0.001	-	<0.001	-

Mean values followed by the different small letters within the same column differ significantly from one another

The results indicate cleaning with chlorine resulted to a considerable reduction of the residue levels of propineb which changed from 9.26±0.09 mg/kg on unclean tomato to a mean of 1.83±0.04 mg/kg on the chlorine cleaned tomato translating to a reduction by 65.1%. Water cleaning reduced the mean residues of propineb from 9.26±0.09 to 5.02±019 mg/kg, translating to 45.8%. The removal of propineb residues are very low with water due to the low solubility of propineb at less than 0.01g/l .The statistical analysis further emphasizes the effectiveness of chlorine cleaning on the removal of propineb residues from tomato vegetable since water cleaning and chlorine cleaning differed significantly.

The mean residues of propineb on sweet pepper reduced from 10.54±0.20 mg/kg in the unclean samples to 9.99±0.18 mg/kg in the water cleaned samples which was a negligible reduction and not significant according to the statistical analysis ($p < 0.001$**,** $\alpha = 0.05$. The low removal of propineb is attributed to the chlorine cleaning reduced the residues to 3.69±0.23 mg/kg which showed a significant difference and translated to a 65.1% reduction on the levels.The effectiveness of of cleaning with chlorine on removal of propineb residues is clearly emphasized by these results. The discrepancies between tomato and sweet pepper are attributed to differences of pH in the homogenates among other reasons. The instability of mancozeb and other dithiocarbamates like propineb increases with increasing pH (Lopez *et* al., 2017). The results correlate favorably with various data from work done by other researchers such as Hwang *et al,* (2001) and Mehmet *et al,* (2014).

4.5.2 Effect of cleaning method on residue levels of mancozeb on vegetables

The results showing the effect on mancozeb residues due to cleaning tomato and sweet pepper are presented in Table 4.8. The levels of mancozeb residue decreased in unclean tomatoes from a mean of 5.23±0.02 to 0.25±0.00 mg/kg in chlorine cleaned tomatoes removing a high amount of mancozeb by 95.2% while water cleaning alone reduced the residues to 4.37± 0.06 mg/kg translating to 16.2% reduction. Mancozeb levels decreased in unclean sweet pepper from a mean of 4.13±0.08 to 1.99±0.01 mg/kg in chlorine cleaned peppers translating to a reduction by 51.8%. The removal of the mancozeb residues are low in sweet pepper when compared to tomato which is attributed to the fact that the solubility of mancozeb is 0.2mg/l at pH 4-5 and 6-8 ,however at pH of 9-10 is higher at 0.3mg/l (Lopez *et al.,* 2017). The tomato homogenate with basic sodium hypochlorite exhibits higher alkalinity levels which hence favor removal of the residues. The residue levels on water cleaned sweet pepper indicated a reduction by 21.6% whereby the residues decreased from 4.13±0.08 to 3.23±0.08 mg/kg.

Table 4.8: Effect of cleaning method on residue levels of mancozeb on vegetables

	tomato		**sweet pepper**	
cleaning treatment	**mg/kg: n=6**	**% reduction**	**mg/kg: n=6**	**% reduction**
Unclean	5.23±0.02^{c}	0.0	4.13±0.08^{c}	0.0
Water only	4.37±0.06^{b}	16.2	3.23±0.08^{b}	21.6
Chlorine	0.25±0.00^{a}	95.2	1.99±0.01^{a}	51.8
p-value	<0.001	-	<0.001	-

Mean values followed by the same small letter within the same column do not differ significantly from one another (P< 0.001, α=0.05)

The statistical analysis shows significant difference on residue levels of mancozeb between cleaning with water and chlorine in both vegetables; however the cleaning of the residues of the fungicides on sweet pepper requires further probing on relationship between interactions of capsaicin with the cleaning agent and the fungicides. The results indicate that sodium hypochlorite (chlorine) reduced mancozeb residues on the vegetables more efficiently than water alone.

The effectiveness of chlorine is further supported by other studies such as Mehmet *et al*. (2014) where a reduction of the residues of mancozeb by 64.66±1.71% on tomato vegetable was achieved with chlorine while Hwang *et al*. (2001) achieved 82% reduction of the residues of mancozeb on apples with chlorine.

4.5.3 Effect of cleaning method on residue levels of ethylenethiourea on vegetables

The results showing the effect of ethylenethiourea (ETU) mean residues due to cleaning tomato and sweet pepper vegetables with water only, with both water and chlorine and then compared to unwashed vegetables are presented in Table 4.9 and discussed comprehensively in this section. The mean residues are compared while at the same time providing percentage reduction of the residues.

Table 4.9: Effect of cleaning method on residue levels of ETU on vegetables

	tomato		sweet pepper	
cleaning treatment	**mg/kg : n=6**	**% reduction**	**mg/kg: n=6**	**% reduction**
Unclean	46.71±0.71^{b}	0.0	2.14±0.02^{b}	0.0
Water only	0.27±0.08^{a}	99.4	1.23±0.06^{c}	39.7
Chlorine wash	0.05±0.00^{b}	99.9	BDL	100
p-value	<0.001	-	<0.001	-

Mean values followed by the same small letter within the same column did not differ significantly from one another (P< 0.001, α=0.05)

From Table 4.9, ethylenethiourea (ETU) residues changed in unclean tomato from a mean of 46.71±0.71 to 0.05±0.00 mg/kg in chlorine cleaned tomatoes which showed a reduction by 99.9%. Cleaning tomatoes with water reduced the mean residues to a mean of 0.27±0.08 mg/kg which indicated a reduction by 99.4% while there was a reduction of (ETU) residues from a mean of 2.14±0.02 mg/kg in unclean sweet pepper to below detection limit (BDL) with chlorine cleaned translating to almost 100%. Cleaning with water only resulted to a reduction of mean residues of ETU in the unclean vegetable from 2.14±0.02 to 1.23±0.06 mg/kg which was a 39.7% reduction. Cleaning with water and cleaning with sodium hypochlorite (chlorine water) differed significantly. A high reduction of ethylenethiourea (ETU) residues on vegetables washed with chlorine was noted. The cleaning treatments from this study lead to conclusions and data which agree with earlier work done as in Knio *et al*, (2000) whereby water wash alone led to reduction of ETU residues on tomato by 70% while in Kontou *et al*, (2004), washing tomato with 0.1% sodium hypochlorite reduced residue levels of ETU to almost 100%.

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

From the results of this study the following conclusions can be made that support the hypothesis.

(i) Residue levels of propineb were found above the recommended levels in tomato from Kutus and Wakulima markets and also in sweet pepper from Kiamutugu market during the wet season while all the samples showing positive results for ethylenethiourea (ETU) had levels above MRL, therefore a high percentage of vegetables consumed in the Kenyan markets have residues of ethylenebisdithiocarbamates and ETU exceeding the recommended MRL.

(ii) Residue levels of mancozeb were not above the MRL in all the four markets in the tomatoes and sweet pepper in both seasons due to its instability in the environment.

(iii) The residue levels of mancozeb and propineb in tomato and sweet pepper were higher during wet season than during dry season while ETU residue levels were higher during dry season than during wet season; which can be due to thermal degradation of mancozeb which yields ETU as metabolite or an impurity.

(iv) The results on the effect of temperature, show an increase in the residue levels of ETU when dithiocarbamates are subjected to various cooking temperatures as high as 100 ºC.

(v) The study found that cleaning vegetables with sodium hypochlorite and rinsing with clean water reduces the residue levels hence making the vegetables safe for consumption.

5.2 Recommendations

The recommendations based on this study are;

(i) Consumers and vendors to embrace cleaning tomatoes and sweet pepper vegetables with chlorine wash (sodium hypochlorite) before eating or selling, respectively.

(ii) It is important to have regular and consistent surveillance of residue levels of dithiocarbamates and ETU from vegetables in our farms, retail outlets and wholesale outlets.

(iii) The consumers to be educated extensively on the methods of reducing pesticide residues on vegetables accompanied with appropriate legislations.

5.3 Recommendations for further study

(i) There is a need for determination of various pesticides in different consumer products used in Kenya.

(ii) There is need for further studies on the effect of pH values of different vegetables on decomposition of different pesticides.

(iii) A survey should be carried out on human body fluids in Kenya to monitor levels of the metabolite ethylenethiourea (ETU).

(iv) Surveillance should be carried out on the levels of ETU on ready foods from the food outlets in Kenya.

(v) Levels of dithiocarbamates and ETU should be analyzed in river water, wells, dams and other water reservoirs from agricultural areas.

(vi) Levels of dithiocarbamates and ETU should be analyzed in animal feed (forder) and animal products from agricultural areas

(vii) The effect of using different soaps and sodium hydrogen carbonate for cleaning vegetables on levels of pesticide residues should be carried out.

(viii) The effect of texture of vegetables and fruits on effectiveness of cleaning on levels of dithiocarbamates and ethylenethiourea.

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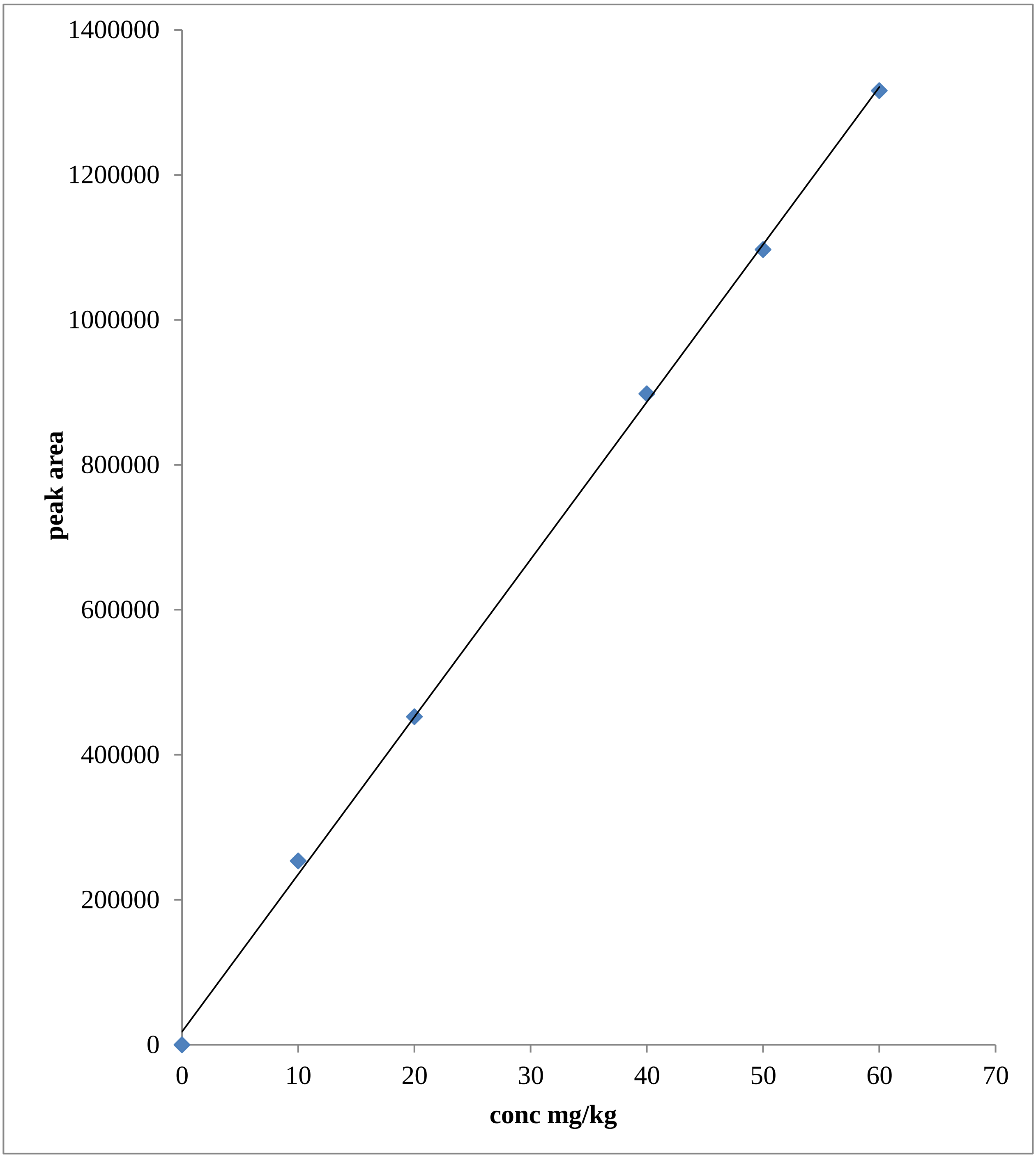
APPENDICES

APPENDIX I

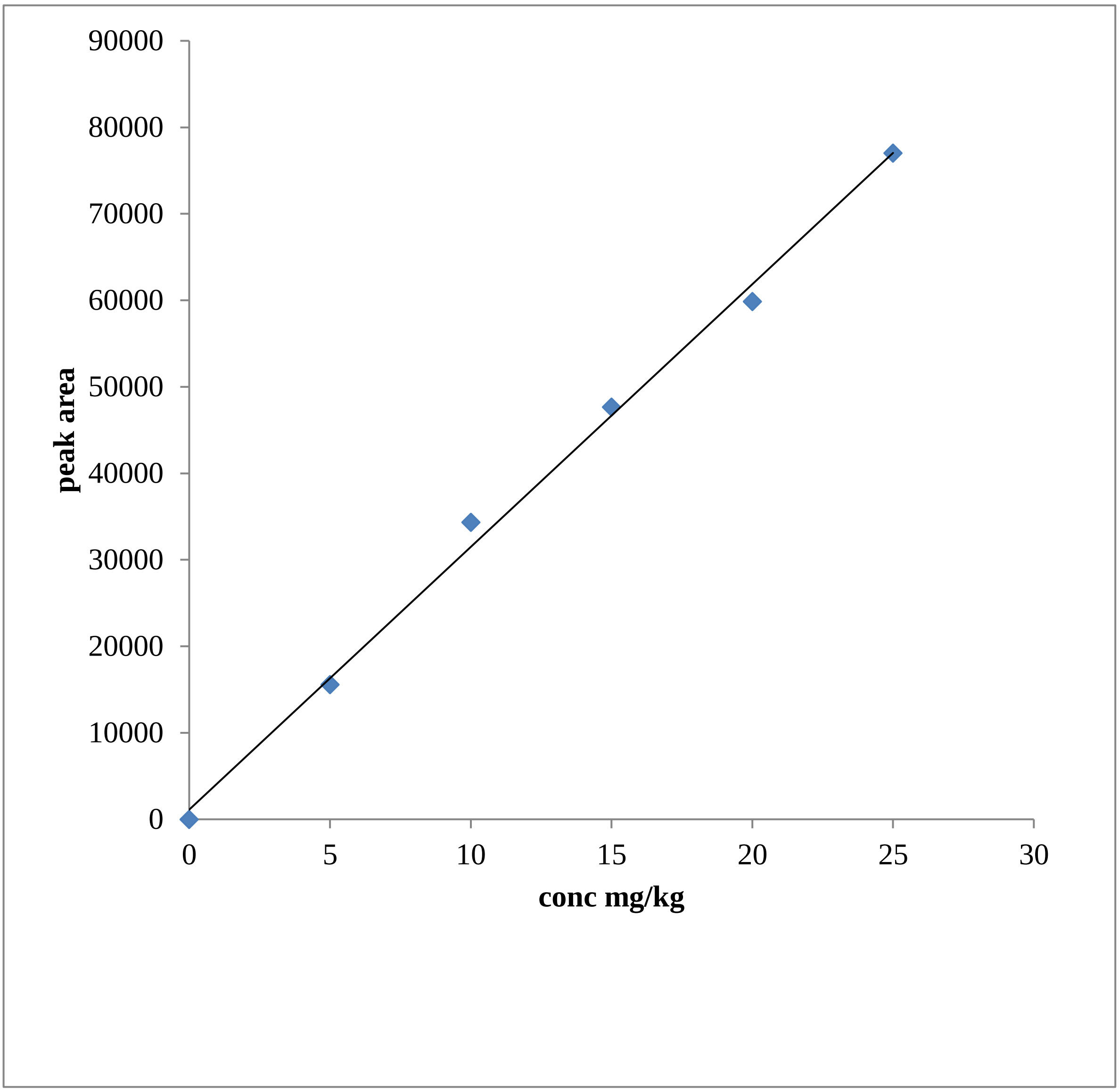
Heating of sweet pepper

APPENDIX II



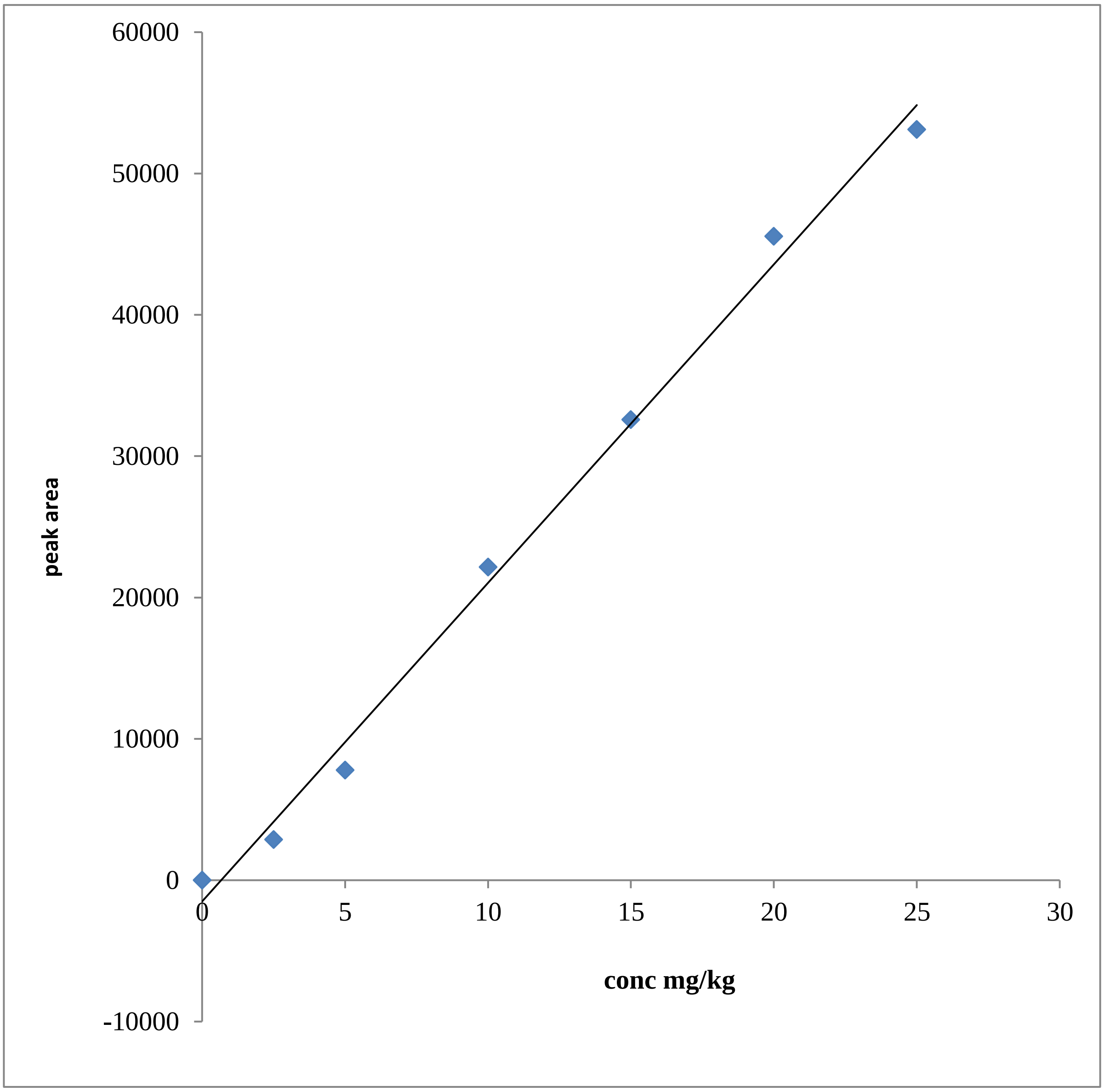
Ethylenethiourea standard curve

APPENDIX III



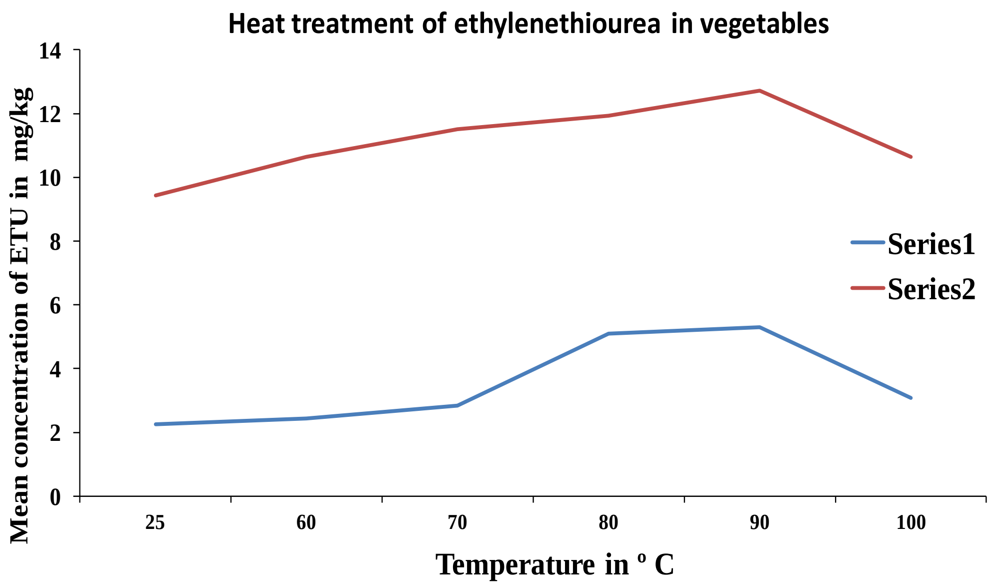
Mancozeb standard curve

APPENDIX IV



Propineb standard curve

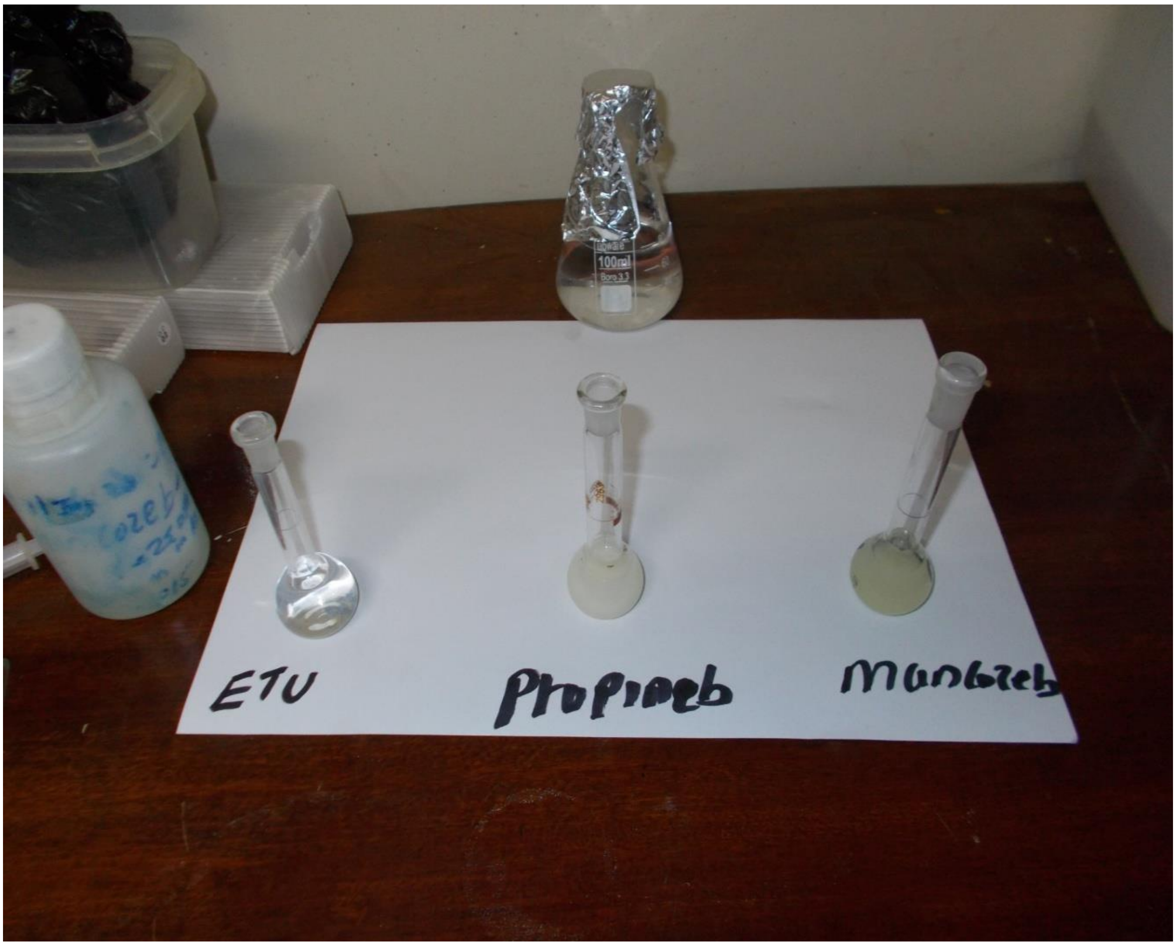
APPENDIX V



Key:Series 1:sweet pepper ,series 2: Tomato

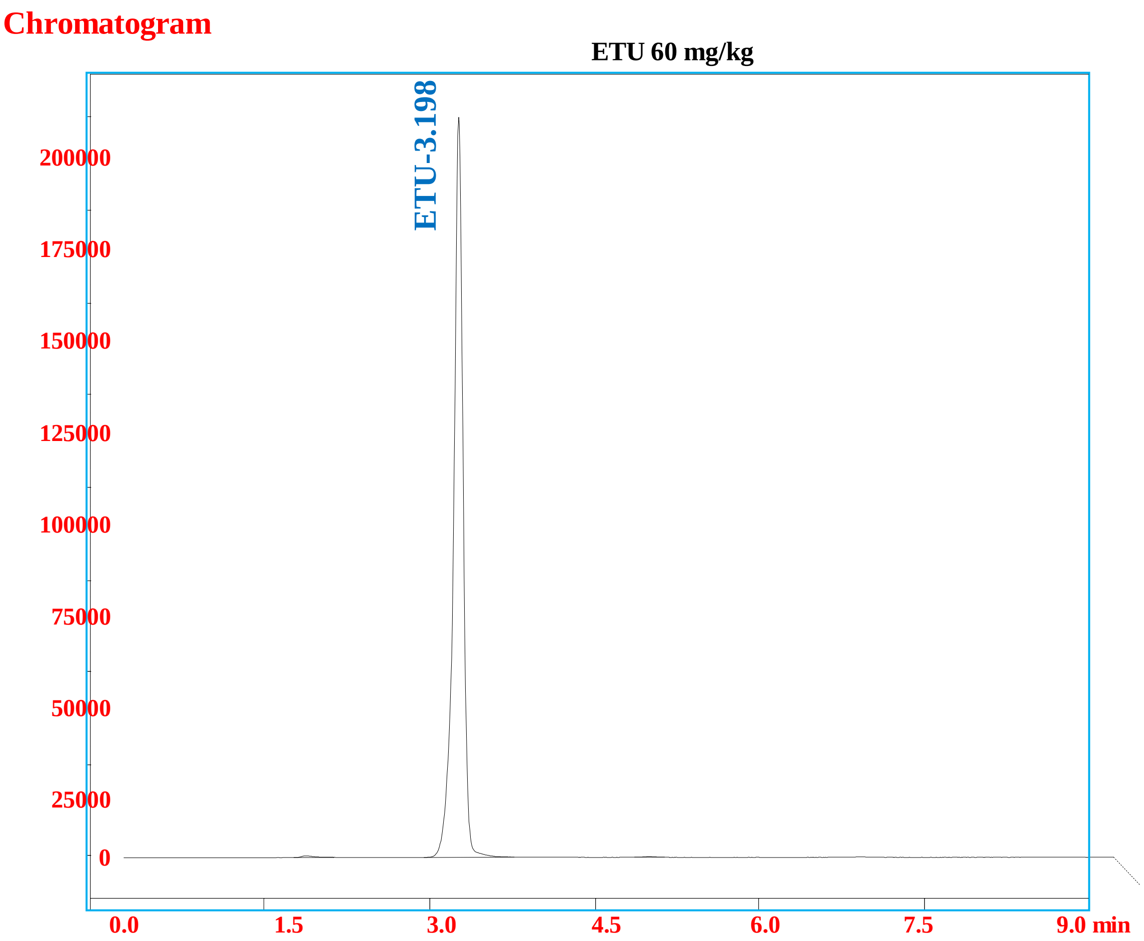
Variation of ethylenethiourea levels with temperature

APPENDIX VI



The stock solutions of standards

APPENDIX VII



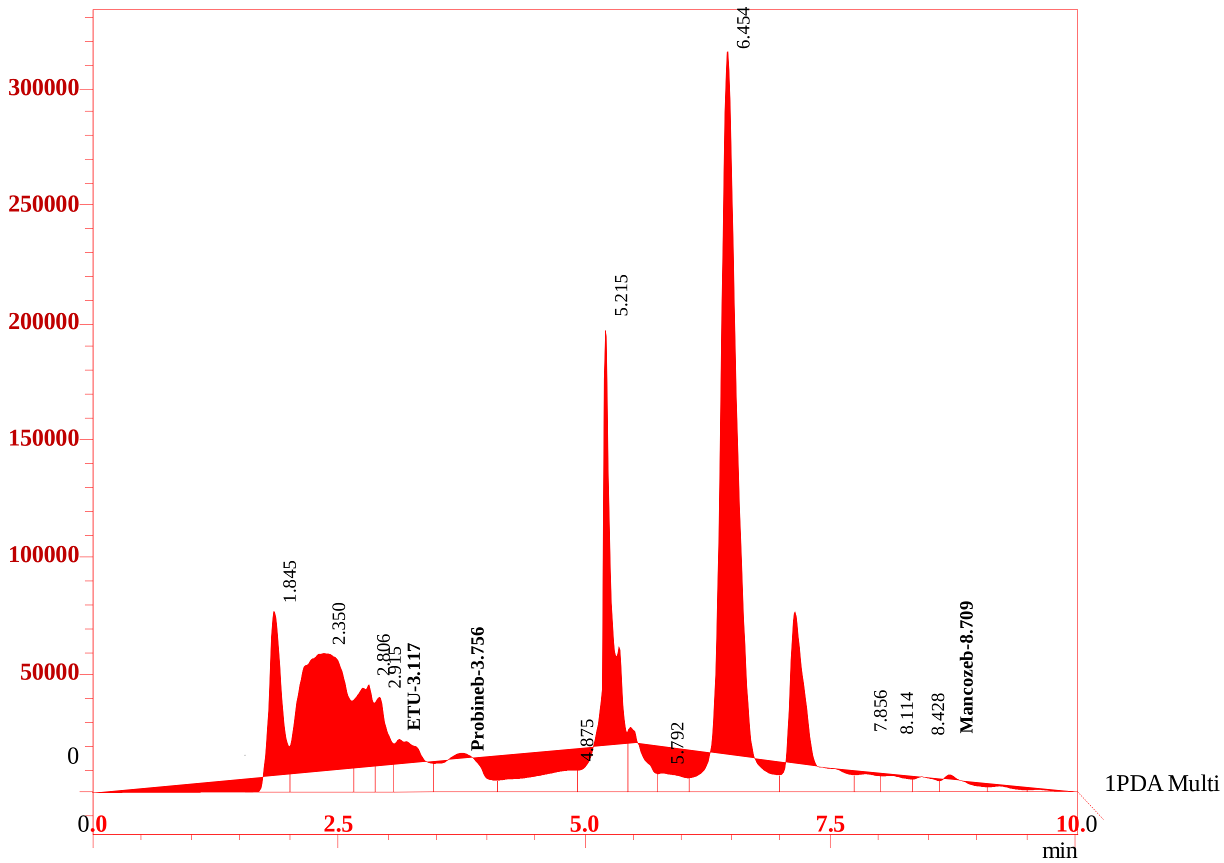
Peak Table

Peak#	Ret. Time	Area	Height	Area %	Height %
1	1.765	17035	2402	1.897	1.88
2	3.198	898015	123769	96.667	96.698
3	5.964	12309	1780	1.325	1.391
Total		928978	127995	100.000	100.000

Ethylenethiourea Standard

APPENDIX VIII

Chromatogram



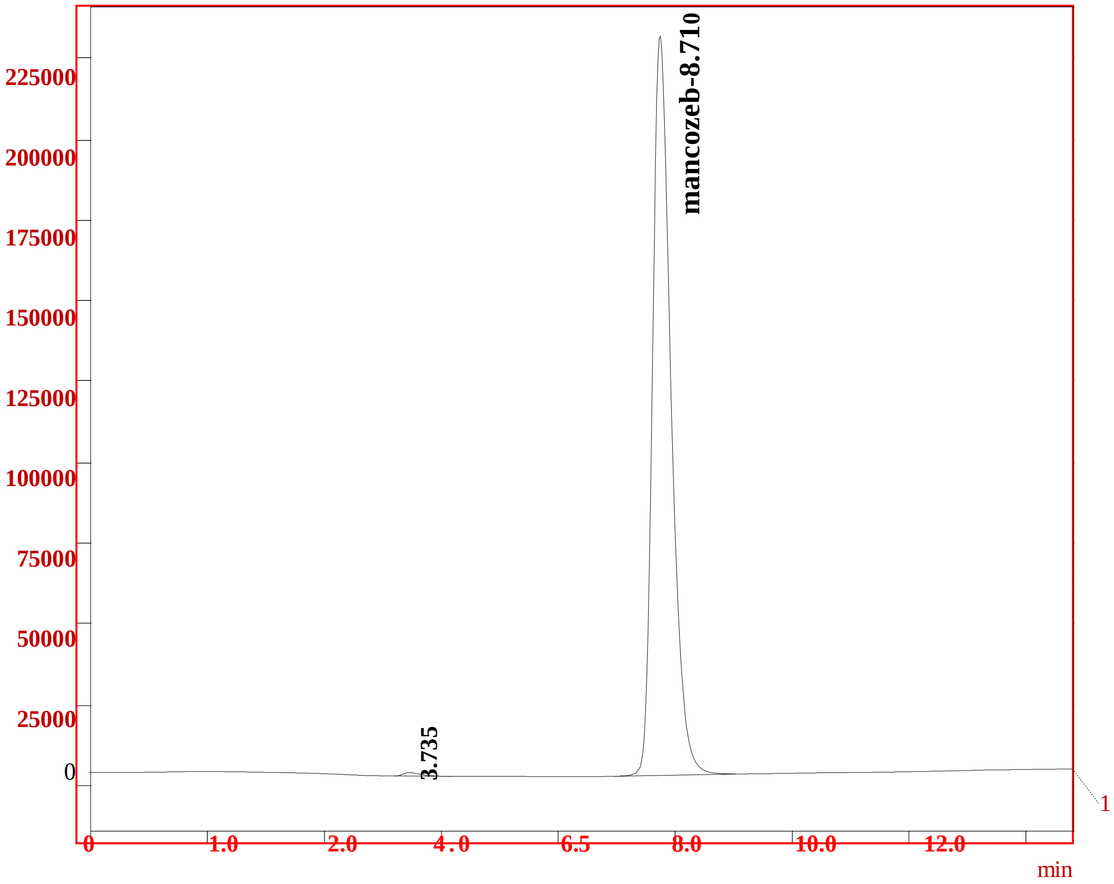
Peak Table

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.117	436399	22457	7.935	5.037
2	3.756	4039	16583	0.073	3.720
3	6.454	3888442	315414	70.704	70.749
4	7.138	1043299	76626	18.970	17.188
5	7.856	114817	7552	2.088	1.694
6	8.709	12606	7187	0.229	1.612
Total		5499602	445819	100.	100.

Tomato sample from Githurai market during rainy season

APPENDIX IX

Chromatogram



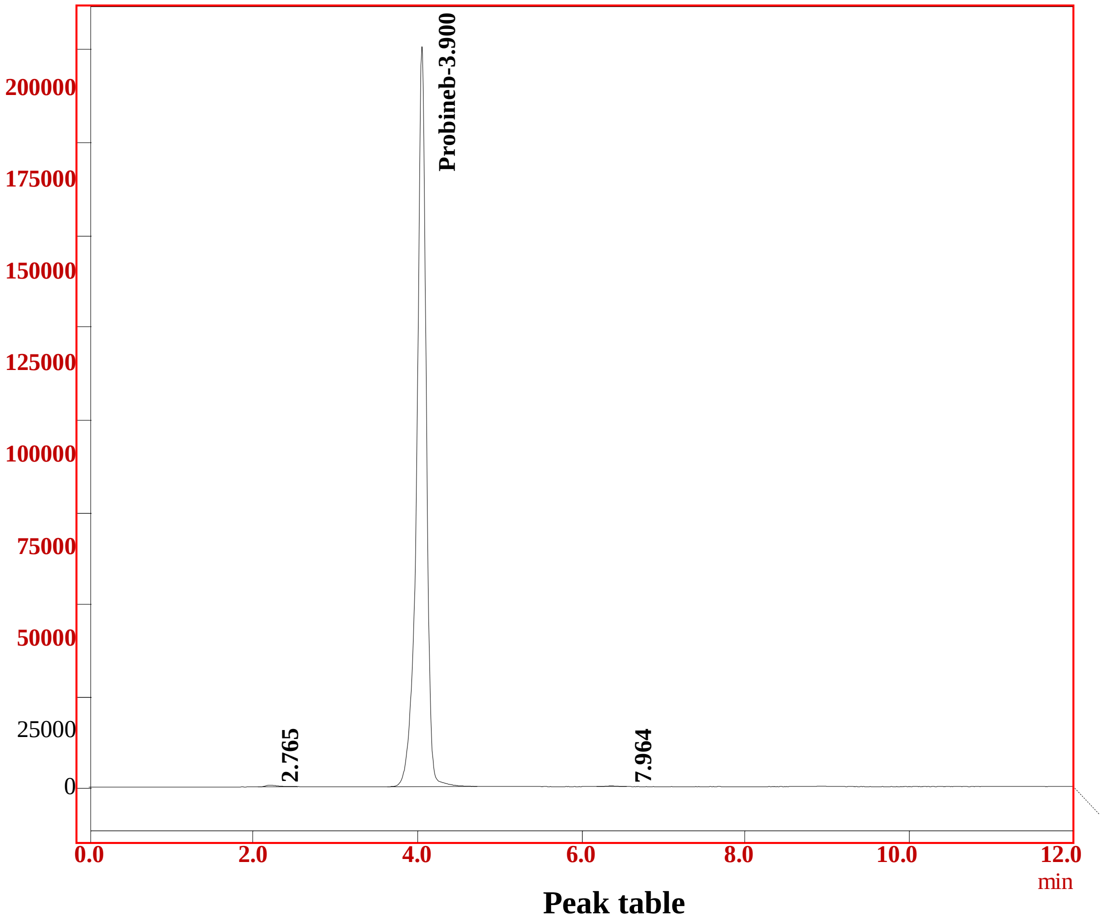
PeakTable

Peak#	Ret. Time	Area	Height	Area %	Height
1	3.735	187	3	0.287	0.287
2	**8.710**	**64679**	**984**	**99.713**	**99.713**
Total		64865	987	100.000	100.100

Mancozeb standard 10 mg/kg

APPENDIX X

Chromatogram

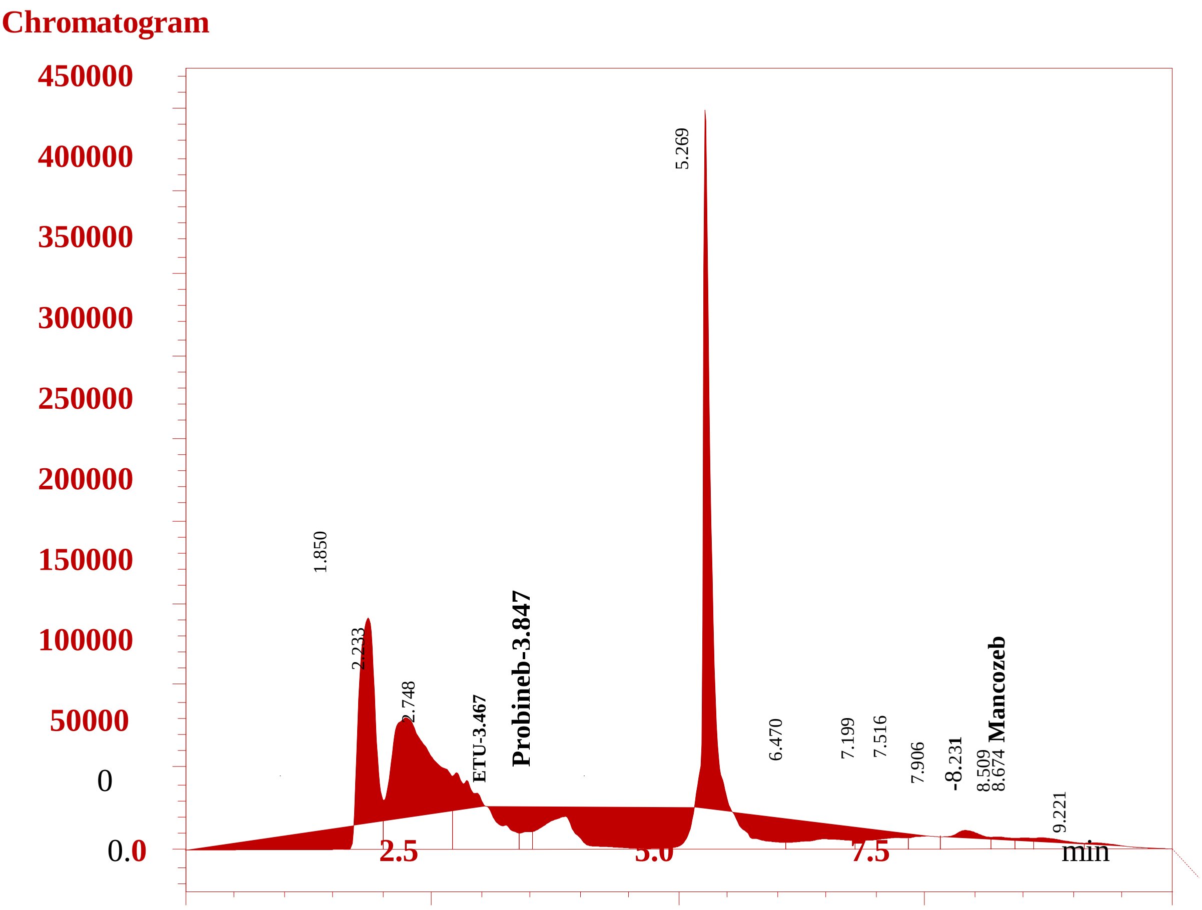


Peak table

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.765	1128	110	8.847	8.995
2	3.900	10918	1066	85.786	85.987
3	7.964	704	78	5.529	5.463
Total		12750	1254	100.000	100.000

Propineb standard 2.5 mg/kg

APPENDIX XI



Peak#	Ret. Time	Area	Height	Area %	Height %
1	1.850	1667744	140412	17.404	17.497
2	2.233	2538471	80003	26.490	9.969
3	2.748	1084424	46543	11.317	5.800
4	3.467	73701	10702	0.769	1.334
5	3.847	5017	1996	0.052	2.482
6	5.269	3092937	448280	32.277	55.861
7	6.470	218732	6151	2.282	0.767
8	7.199	193472	6857	2.019	0.854
9	7.516	148350	7811	1.548	0.973
10	7.906	270775	11276	2.826	1.405
11	8.231	106608	7317	1.113	0.912
12	8.509	75528	6697	0.788	0.835
S13	8.674	19326	6879	0.202	0.857
14	9.221	87497	3650	0.830	0.455
Total		9582581	784573	100.000	100.00

Sweet pepper sample from Kiamutugu during dry season

APPENDIX XII

THE HPLC PROGRAM

Time	module	Action	Value
0.00	pumps	B Conc.	0
0.01	pumps	B Conc.	0
0.00	pumps	C Conc.	0
0.01	pumps	C Conc.	0
0.00	pumps	D Conc.	0
0.01	pumps	D Conc.	0
2.00	pumps	B Conc.	0
2.01	pumps	B Conc.	30
2.00	pumps	C Conc.	0
2.01	pumps	C Conc.	33
2.00	pumps	D Conc.	0
2.01	pumps	D Conc.	37
6.00	pumps	B Conc.	0
6.01	pumps	B Conc.	0
6.00	pumps	C Conc.	0
6.01	pumps	C Conc.	0
6.00	pumps	D Conc.	0
6.01	pumps	D Conc.	0
10.00	pumps	D	0
10.01	pumps	CONTROLLER	STOP

Key: Pump A: 95% 0.1M sodium dodecyl sulphate + 5% acetonitrile,

Pump B: 0.1 M sodium dodecyl sulphate, Pump C: Methanol, Pump D: Acetonitrile