ASSESSMENT OF THE LEVELS OF SOME TOXIC AND ANTINUTRITIONAL SUBSTANCES IN KENYAN COFFEE PULP

BY

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A Thesis submitted in partial fulfillment for the degree of Master of Science.

Kenyatta University

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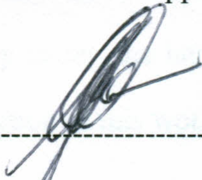

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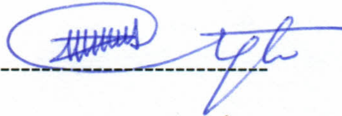

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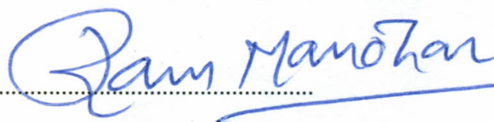

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DEDICATION

This work is dedicated to my father, the late Kiptoo Kimalbei Karyena who could not live to witness the completion of this work.

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ABSTRACT

Every year, the Kenyan coffee industry produces over 350,000 tons of clean coffee and similar quantities of associated by-products (coffee pulp and coffee husks). These by-products are often damped into nearby streams causing serious pollution problems. Solution to this problem calls for the evaluation of available alternative uses for the by-products and safer disposal methods. This work explored the possibility of using Kenyan coffee pulp as animal feed. Levels of some toxic and antinutritional substances in coffee pulp samples collected from fifteen coffee processing factories located in Thika and Kiambu districts were determined.

The levels of caffeine, total tannins and potassium were in the range 0.22-0.70, 1.31-1.56 and 0.9-4.21%w/w on dry mass basis respectively. Levels of Cu, Zn, Mn and Fe were in the range between 4.90-213.30, 11.23-28.03, 3.49-119.29 and 12.53-452.8μg/g respectively. Among the pesticides, only chlorothalonil was detected in most samples at appreciable amounts (0.02-1245μg/g). After ensiling for a period of four (4) months, the mean percentage losses ranged 30-45.59% for caffeine, 5.56-42.50% for total tannins and 77.28-100% for the pesticide residues. From these figures, it is recognized that ensiling is a significant detoxification method.

The distribution of caffeine and tannins seemed to depend on the prevailing environmental conditions. Coffee pulp from well managed farms had higher levels of caffeine while higher levels of tannins were reported in coffee pulp from agro-ecological zones that are more exposed to fungal diseases or insect pests. Coffee pulp from the

same agro-ecological zone were close in their K, Zn, Mn and Fe contents, implying that soil characteristics plays an important role in the accumulation of these metals in the pulp. The distribution of copper was rather erratic. Copper sprays as well as spray programmes contribute greatly to the amount of copper in the pulp.

The results of this study show that coffee pulp is contaminated with toxic and antinutritional substances. However, the levels of caffeine, tannins and the pesticide residues were reduced significantly upon ensiling, though not completely. The levels of the metals reported in this work are within the levels acceptable to most animals and cannot be considered a major setback in the prospect of converting coffee pulp to animal feed. Although the levels of pesticide residues reported in the fresh pulp are within the levels considered toxic to most animals, ensiling reduced the levels to those below the maximum residues levels (MRL's) in or on animal feeds and feedstuffs as recommended by FAO/WHO (1998). Therefore it appears that coffee pulp can at best be initially used at well-rationed supplemental levels with other forages or feed components as more effective methods of detoxification are explored.

CHAPTER ONE

1.0. *Introduction.*

1.1. Coffee- Origin, Description and Distribution.

The coffee tree is indigenous to Ethiopia but was first cultivated and used as a beverage in Arabia (Smith, 1985). It belongs to the family *Rubiceae* and the genus *Coffea.* The coffee tree can grow to 10 -20 m high but is usually pruned to a height of 2 -3 m to facilitate harvesting and maximize yields. There are about 500 species of the genius *coffea* but those of commercial importance are:

(i) *Coffea arabica*, known in trade simply as arabica coffee and accounts for 76% of the world's commercial coffee (Graaf, 1986).

(ii) *Coffea canephora*, commonly referred to simply as robusta in trade and accounts for 23% of the world's commercial coffee (Graaf, 1986).

(iii) *Coffea liberica*, simply known as liberica in trade and accounts for only 1% of the world's commercial coffee (Graaf, 1986).

The economics of coffee production lies in the coffee berry or fruit. The coffee berry shows four anatomical fractions: the coffee bean proper (endosperm), the hull (endocarp), a layer of mucilage (*mesocarp*) and the pulp (epicarp). The coffee pulp, also identified, as coffee fruit without seeds constitutes 40- 43% of the fruit on a fresh mass basis (Bressani, 1979).

Coffee enjoys a subtropical, almost temperate climate that is free from frost. The rainfall should be well distributed with a definite dry season. Temperatures of between 17-25^{0} C are favourable. Coffee is grown commercially in Africa, the Americas, and Asia but not in Europe because of unfavourable weather conditions. Brazil and Colombia are

the world's leading producers (Marshall, 1987). In East Africa, Arabica coffee grows best in highland areas lying 1400m-1900 m above sea level with an annual rainfall of 1500mm-2250mm. Robusta grows in lower and drier areas (Wrigley, 1988).

Arabica Coffee prefers deep, well-drained loamy soils, slightly acidic, rich in humus and exchangeable bases such as potassium. The best coffee soils in Kenya are the Kikuyu red loam soils that are of volcanic origin, acidic and low in phosphorous and lime. The ideal pH range is 5.2-6.2, but it can be as wide as 4.6-7.5 (Wrigley, 1988).

In Kenya, the coffee industry mainly produces arabica coffee, which is predominantly grown in the Central and Eastern provinces. Central and Eastern provinces alone account for about 80% of the total acreage under coffee, while Western, Rift Valley and Nyanza provinces account for about 19%. The other 1% is grown in the Taveta area of Coast province. Robusta is grown in the lower and drier areas of Nyanza province. Arabica is of higher quality and commands better prices in the market (Mburu and Mwaura, 1996).

1.2. The General Importance of Coffee.

Coffee is a popular beverage throughout the world, partly for its agreeable taste and aroma and partly for its stimulating effect for which caffeine is largely responsible (Battig, 1987). Caffeine has been used therapeutically in the treatment of apnoea, as a bronchial and cardiac stimulant, in the treatment of skin disorders and migraine headaches (Battig, 1987). It has also been used as an analgesic, a diuretic, a weight control aid, for allergy relief preparations and alertness compounds (Battig, 1987).

Coffee is of prime economic importance to the producer countries. The major producer countries are third world countries whose economies are primarily agricultural and

therefore coffee is a good source of foreign exchange. Most of the coffee beans are exported to markets in the developed world where they fetch good prices (Marshall, 1987). Brazil is the world's largest coffee producer, accounting for 21.1 % of the total production followed by Colombia and Indonesia at 13.9 and 7.3 % respectively. Kenya leads Africa at about 1.6 % of the total world coffee production (FAO, 1996).

Coffee has remained one of the most important agricultural products of Kenya's agriculture, since its introduction in 1900. It is estimated that about 70% of the national labour force is employed in the agricultural sector, one third of which is absorbed in the coffee industry (Kenya Coffee, 1998). In terms of foreign exchange earnings, coffee is ranked third after tourism and tea, contributing about 20% of the total foreign exchange earnings. However, there is emerging competition from the horticultural and floricultural industry (Kenya Coffee, 1998). With fluctuating coffee prices in the international market, climatic changes, pest and disease problems, the contribution of coffee in the total export earnings in Kenya has also been changing.

1.3. Coffee Pests and Diseases.

The coffee tree is prone to attack by many pests and diseases, which cause severe losses of the crop. In addition their control by use of chemicals raises the total cost of coffee production, negatively impacting on the economic returns (Willson, 1999). Robusta coffee is noted for its resistance to pests and diseases though its beans are of poorer quality than those of arabica, and fetch comparatively lower market prices. Thus the pests and disease described in this section apply mainly to arabica coffee (Waller, 1987).

Coffee diseases are caused by pathogenic microfungi and occasionally by bacteria and some viruses. The common diseases include; coffee berry disease (CBD), coffee leaf

rust, brown blight of leaves, bacterial blight, sooty mould, warty disease, berry blight, e.t.c. Of these, CBD and leaf rust are by far the most important since they adversely affect coffee yields as well as quality (Waller, 1987).

CBD is caused by the fungus *Colletotrium Coffeanum*. It infects growing berries, causing them to rot and be shed from the plant before the beans have formed. In ripening cherries, it causes the pulp to stick to the beans, making processing by the wet method difficult. It can also impart taint characteristics in coffee aroma, a consequence of continued microbial activity inside the bean, which produces off-flavours. CBD infestation is favoured by wet conditions as rainfall is required for spore production, dispersal and growth. Currently CBD is limited to East, West and Central Africa (Waller, 1987).

Coffee leaf rust is caused by the fungus *Hemileia Vastatrix*. It is the most widespread coffee disease, though less harmful than CBD in terms of the extent of damage it can cause to coffee production. It has been reported in all the coffee growing regions of the world. The fungus forms orange powdery spots on the underside of the leaves. The leaves will eventually fall if not treated. The defoliation will negatively affect the next harvest as a result of the stress subjected to the coffee tree (Waller, 1987).

Coffee grows in climates without severe weather extremes, consequently a large variety of insects and other pests can survive from year to year. This also applies to the parasites and predators; therefore effective natural biological control occurs for most of the pests. The pests that are commonly known to feed on coffee include; leaf miners, mites, coffee stem borers, beetles, and cutworms (Willson, 1999).

1.4. Some common Pesticides used in the control of coffee pests and diseases.

The term 'pesticide' refers to certain chemical substances and microorganisms (bacteria, fungi, viruses and mycoplasmas) used to control pests. The term 'pest' is used in a broad sense to include animals, microorganisms and plants (weeds) as well as insects. Pesticides include fungicides, herbicides, insecticides, miticides, nematocides and miscellaneous compounds such as wood preservatives, plant growth regulators, soil sterilants, animal and bird repellants and sensory biocides (Pesticide manual, 1991).

Coffee is a capital-intensive cash crop. The coffee trees have to be protected against pests and diseases throughout the year and a wide range of insecticides, fungicides and herbicides, which may be organic-based or inorganic-based, are available in the market for this purpose. The pesticides may be classified as contact (protectant) or systemic. Contact pesticides are deposited on the plant surfaces and kill the pest on contact, thereby preventing the host plant from attack as well as preventing the pest from infected plants moving to healthy ones nearby. Their disadvantage is that they are susceptible to the effects of weathering (rain, wind, and sunlight) over long periods leaving the plant unprotected and therefore have to be applied onto the susceptible parts of the plant fairly frequently. Secondly, they cannot kill pests that are well established in the plant tissues since they cannot be translocated through the plant (Hassall, 1990).

The systemic pesticides effectively penetrate the plant cuticle and move through the plant vascular system. This property confers fundamental advantages to this class of pesticides. Firstly, they are little affected by weathering and are applied less frequently. Secondly they can kill pests that are well established in the plant tissues and will also

confer immunity to new plant growth (Hassall, 1990). However, pest resistance is often encountered in the use of systemic pesticides and there is the danger of phytotoxicity when pesticide levels accumulate in the plant (Waller, 1987).

1.4.1. Fungicides.

Most of the diseases that are a problem in coffee production are of fungal origin. A number of fungicides, both inorganic and organic-based are available for the control of CBD and leaf rust. They are applied as sprays or dusts to the plant foliage. The ones that are commonly used in Kenyan coffee industry include the wettable powders (WP), emulsifiable concentrates (EC) or soluble concentrates (SC) of bordeaux mixture, $CuSO_4.3Cu(OH)_2$; copper oxychloride, $CuCl_2. 3Cu(OH)_2$; basic copper carbonate, $Cu(OH)_2.CuCO_3$; cuprous oxide (red oxide), Cu_2O; burgundy mixture, $CuSO_4.3CuCO_3$ and chestnut compound, $CuSO_4.NH_4OH$. The organic based fungicides include; chlorothalonil (2,4,5,6-tetrachloroisophthalonitrile), captafol, (N- (1,1,2,2-tetrachloroethylthio) cyclohex-4-ene-1,2-dicarboximide), captan (N-(trichloromethylthio)cyclohex-4-ene-1,2-dicarboximide), dithianon (5,10-dihydro-5,10-dioxonaphtho[2,3-b]-1,4-dithi-in-2,3-dicarbonitrile), triadimefon (1-(4-chlorophenoxy)-3,3-dimethyl-1-(1H,-1,2,4-trizol-1-yl)-2-butanone), anilazine (4,6-dichloro-N-(2-chlorophenyl)-1,3,5-triazin-2-amine), rochloraz (N-propyl-N[2-(2,4,6-trichlorophenoxy)ethyl]imidazole-1-carboxamide) and hexaconazole (RS)-2-(2,4-dichlorophenyl)-1-(1H-1,2,4-triazol-1-yl)hexan-2-ol) (Kenya Coffee, 1997). Table 1 gives the rates of application of the various formulations for the fungicides that are commonly used in Kenya.

In Kenya, copper-based fungicides are widely used because of their relatively low cost and wider biological activity (they are used to control bacterial blight as well as against a wide variety of fungi). They are popularly used by small-scale farmers who cannot afford the more expensive organic based fungicides. The organic based fungicides have a narrow biological spectrum and are more effective at lower concentrations. Some of them are systemic and confer more effective immunity to the plants for longer periods (Hassall, 1990). Consequently, the organic based fungicides are applied at lower rates (Table 1), implying that they are cost effective in the long run.

1.4.2. **Insecticides.**

The insecticides commonly used in the control of insect pests in Kenyan coffee plantations are mostly organophosphates. Use of organochlorine insecticides has been discontinued due to environmental concerns. The insecticides available in the Kenyan market include the emulsifiable concentrates of fenthion (O,O-dimethyl - [4-(methylthio)-m-tolyl]phosphorothioate), ethion (O,O,O',O`-tetramethyl SS`-methylene bis (phosphorothioate), chlorpyrifos (O,O-diethyl-O-(3,5,6-trichlor-2-yridyl) phosphorothioate), fenitrothion (O,O-dimethyl O-(3-methyl-4-nitrophenyl)phosphorothioate) and dimethoate (O,O-dimethyl S-methylcarbamoylmethyl phosphorodithioate).

There are no specific spray programs to be followed in the case of insecticides because they are not recommended for use as a preventive measure. So when they have to be used, it should be in such a way that they have the minimum effect on beneficial insects and other non- target organisms. Insecticides are recommended for use only when the insect population grows to a size that if unchecked is likely to cause losses (economic

threshold). After many years of research, the Coffee Research Foundation has come up with the following insect populations per tree after which the appropriate insecticide can be applied; Antestia; 2, Capsids; 4,Giant looper; 20-30 caterpillars per tree, Leaf miner; over 35 moths per tree, Leaf skeletonizer; 20-30 caterpillars per tree, Thrips; 2-3 per leaf when there is rain or 1-2 per leaf during times of drought (Kenya Coffee 1996).

Table 1.Tested and recommended fungicides for CBD and leaf rust control in Kenya. (Kenya Coffee, 1997).

Common name	Formulations	Rate kg/Ha (L/Ha)	Interval between sprays (weeks)
Cuprous Oxide (Red Oxide)	50% Cu WP	7.7	4
	75% Cu WP	3.8	4
Copper Oxychloride	50% Cu WP		
(green copper)	24%Cu	7.7	4
	Flowable	14.0	4
Copper sulphate+ lime	25% Cu WP		
	50% Cu WP	11	4
Cupric hydroxide	40%Cu	7.7	4
(blue copper)	granules	7.15	4
Chlorothalonil/Copper	25/30 WP	7.7	4
Anilazine/Copper	17/30 WP	9.0	4
Chlorothalonil	75% WP	4.4	4
	50% SC	4.4	4
	72% SC	3.5	4
Dithianon	75% WP	3.3	4
	50% SC	2.2	4
Anilazine	75% WP	4.4	4
	48% WP	6.0	4
Captafol	80% WP	4.4	4
Triamedifon	50% WP	2.2	Curative
Hexaconazole	50% EC	2.2	Curative

Key
WP = Wettable powder
SC = Soluble concentrate
EC = Emulsifiable concentrate

1.5. Coffee processing methods and their by-products.

Preparation of coffee beans for the market follows one of two distinct processing methods; the dry and wet methods. The wet process involves a preliminary washing where inferior quality cherries are removed. The washed cherries are then subjected to a mechanical depulping operation with the help of water. The beans so obtained are fermented in concrete tanks for up to three days. In this process enzymes break down the mucillage adhering to the parchment. After fermentation, the parchment is sun-dried then the outer casing (parchment) is removed mechanically using a dehulling machine. The beans thus obtained are ready for roasting or export (Mburu, 1995).

In the dry or natural processing method the beans are first dried in the sun followed by a dehulling process. It is limited to drier areas where the large amounts of water required for the wet process may not be available. Robusta coffee is preferably processed by the dry method because its quality is not appreciably improved by the wet method.

In Kenya the wet process is widely followed in processing arabica coffee (Mburu, 1995). This process yields coffee pulp, mucillage and wastewaters and later the coffee husks or hulls as by-products. The coffee pulp is the most abundant, constituting about 28% on dry weight basis (Bressani, 1979b). Table 2 shows the material balance for the various by-products of coffee processing.

Coffee processing wastes are often contaminated with pesticides and present serious environmental problems. It is desirable to find safer disposal methods and possibly profitable alternative uses for these by-products. Among these by-products coffee pulp has

the greatest potential of economic utilization. The possible uses of coffee pulp that have been studied include;

Table 2. **Material balance of coffee processing** (Bressani, *1979*b).

Material	% on fresh weight	% moisture	% on dry weight
Coffee berry	100	65.5	100
Coffee pulp	43.2	77.0	28.7
Coffee beans	56.8	56.0	72.2
Mucillage	-	17	4.9
Parchment coffee	-	50	65.2
Coffee hulls	6.1	32.0	11.9
Commercial Coffee beans	38.9	51.0	55.4

(i) **Animal feed.**

Coffee pulp has high crude protein content (3.73%), carbohydrates (56.03%) and mineral content such as sodium, potassium, magnesium, boron, iron, zinc etc., suggesting that it has high nutritive value for animal nutrition. However, the presence of antinutritional factors is a major limitation (Melich, 1965, Bresssani, 1979). These are natural substances that occur widely in animal feedstuffs or forages. They generally interfere with the normal nutrition of farm animals. Although the evidence is not conclusive, caffeine, tannins and other polyphenols and a high potassium content in coffee pulp have been implicated to be responsible for its antiphysiological effects (Bressani and Braham, 1979).

According to a study by Bressani, (1979a) experimental evidence suggested that a well processed coffee pulp can be fed up to 30% dry weight for dairy and beef cattle, up to 16% for swine and up to 5% for poultry, in well-balanced diets. When fed at levels higher than these, they reduce feed intake with low feed conversion efficiencies.

(ii) **Organic Fertilizer.**

The high nitrogen and potassium content of coffee pulp has presented an opportunity for use of coffee pulp as an organic fertilizer in coffee plantations. In most cases the fresh pulp is applied directly from the pulpers to the coffee plantation. A second approach is to dry the pulp in the sun before application. Castro (1960) has indicated that 45kg of dried coffee pulp is equivalent, on the basis of chemical composition, to 4.5kg of the 14-3-37 NPK inorganic fertilizer. Other experiments have shown that coffee pulp is a valuable organic fertilizer especially for the coffee tree (Bressani, 1979b).

Currently, this is the only environmentally friendly disposal method used in most coffee processing factories in Kenya. With the growing emphasis on organically produced foods, the use of coffee pulp as an organic manure is likely to gain popularity and the scope of use may widen to include other crops like vegetables.

(iii) **Extraction of Caffeine.**

Caffeine has a variety of uses (Battig, 1987). It is added to most soft drinks as a stimulant. Coffee pulp contains about 0.7% caffeine on dry mass basis (Clifford and Ramirez, 1991), which if extracted, can boost the economic value of coffee production since it has a relatively high market price.

Studies have been carried out on extraction of caffeine from coffee pulp. Various solvents and methods of extraction were tested on fresh and dried coffee pulp such as

extraction with ethanol and water. These methods achieved at least 70% extraction of the caffeine (Murillo, 1974). However, no practical food applications for the extraction of caffeine have been reported

(iv) **Production of Biogas, Ethanol and Vinegar.**

Coffee pulp has a high organic matter content. Its fermentation products can been studied for possible production of biogas, alcohol, wine and vinegar. Hutchison (1965) found that coffee pulp is a good biogas producer, but only when mixed with cattle manure in a batch digester. The rather unsatisfactory results from coffee pulp alone could be due to the large particle size of the pulp. Adams (1980) estimated that a factory producing one ton of clean coffee per day would also produce about $130m^3$ of biogas from the pulp, an equivalent of 100 litres of petrol in terms of fuel value.

Despite its high content of soluble carbohydrates (56.03%), extraction of alcohol from coffee pulp has not been economical. Alcoholic fermentation of the juice from fresh pulp by yeast gives a solution containing 2.5-5.0%v/v ethanol (Adams, 1980). It therefore means that more sugar would have to be added to the juice. The production of vinegar from coffee pulp has been successful in Brazil where factories have been able to produce between 150-250 litres of vinegar per day using wet processed pulp (Adams and Dougans, 1981).

1.6. Metals in plant and animal nutrition.

The metals potassium, Manganese, copper, iron and zinc are essential elements in both plant and animal nutrition. Potassium is a macroelement in plants, required for vegetative growth. The other metals are essential at trace amounts but at high levels they

become toxic (Willson, 1985). Most soils supply ample amounts of these elements and deficiency symptoms are rarely reported (Willson, 1985). The table 3 shows the optimum levels in the leaves of coffee required for normal growth.

Table 3. **Critical levels of some mineral nutrients in coffee leaves (Willson, 1985).**

Species	%K (dry mass)	Cu ($\mu g/g$)	Zn($\mu g/g$)	Mn($\mu g/g$)	Fe($\mu g/g$)
Arabica	2.10-2.60	7-20	15-30	50-100	70-200
Robusta	1.80-2.20	20-40	15-30	35-70	70-200

Elevated levels of potassium has been implicated for the antinutritional effects observed in animals fed with coffee pulp (Bressani, 1979). Although the other metals are generally of low mammalian toxicity, their contribution to animal nutrition is critical. However, the extensive use of copper-based fungicides in the coffee industry implies that there is a possibility of the copper tolerance level in coffee pulp feed being exceeded. Pigs require much higher concentrations of copper in their diets (200-250μg/g) to stimulate growth (Miller *et al.*, 1979), therefore elevated levels of copper in coffee pulp would be important to swine nutrition. A more detailed review on these metals will be provided in the next chapter.

◈ CHAPTER TWO

2.0. *LITERATURE SURVEY.*

2.1. Introduction:-

Coffee pulp is a potential pollutant but its inherent chemical composition shows a great possibility of economic utilization. This has been the focus of research by several workers, not only because it is the most abundant and troublesome coffee processing by-product, but also because it presents the greatest opportunities for economic utilization. Analysis of the nutrient and chemical composition of the pulp by several researchers (Wallis 1958, Melich, 1965, Adams, 1980 and Njoroge 1997) shows that it has high nutritive value. Table 4 below gives a comparison of the nitrogen, phosphorous and potassium contents of coffee pulp and other animal and plant by-products.

Table 4. Nutritional value of Coffee pulp and other animal/plant by-products. (Njoroge,1997).

Material	% N	% P_2O_5	% K_2O
Coffee pulp	3.73	0.4	6.51
Coffee husks	0.48	0.07	0.4
Cattle manure	2.5	1.12	6.70
Maize stover	2.11	0.35	1.95
Napier grass	1.51	0.62	4.23
Poultry manure	3.54	3.24	1.55

With a view of alleviating disposal problems as well as developing an economically viable product, several possible alternative uses have been studied (Bressani, 1979b). Figure 1 shows the processing methods and the possible economic utilities of coffee pulp.

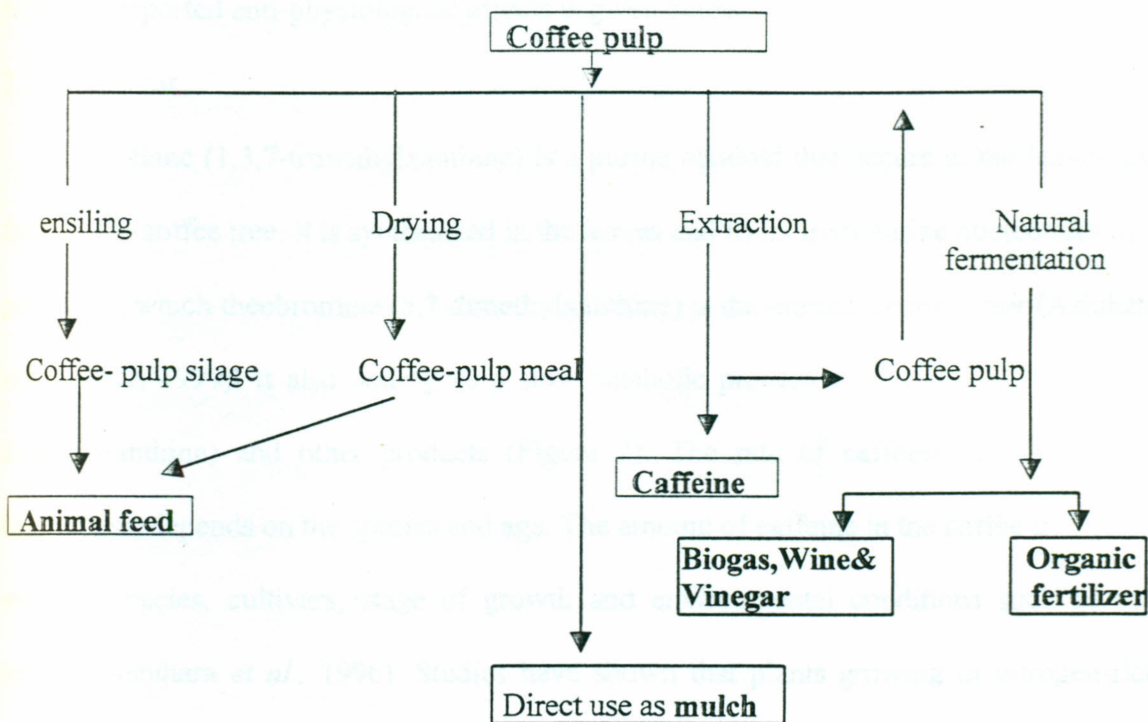


Figure 1. Possibilities for the utilization of Coffee pulp (Bressani, 1979b).

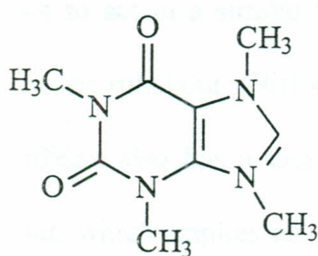
2.2. Antinutritional substances.

Attempts have been made to utilize coffee pulp as a feed for swine, cattle and poultry. However, little success has been achieved due to the presence of anti-physiological substances. These are substances that naturally occur in forage, and which when present at high levels can produce deleterious effects on animals, such as reduced growth rate, poor feed conversion efficiencies, hormonal changes and occasional organ damage. These pathological changes however rarely lead to death. The mechanisms of action may include protein precipitation or complexation, reduction of solubility or absorption of mineral

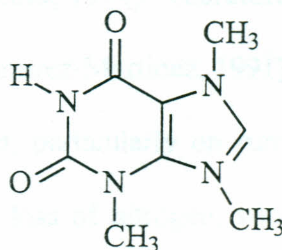
elements and inactivation of certain vitamins (Chubb, 1983). The exact mechanisms of action for most of these substances have not, however, been fully understood. (Clifford, 1991). A brief description of the anti-nutritional factors occurring in coffee pulp, together with their reported anti-physiological effects is given below.

2.2.1. Caffeine.

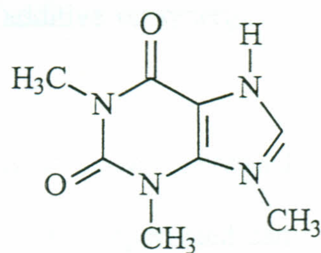
Caffeine (1,3,7-trimethylxanthine) is a purine alkaloid that occurs in the leaves and fruits of the coffee tree. It is synthesized in the leaves and fruits from purine nucleotides by a pathway in which theobromine (3,7-dimethylxanthine) is the immediate precursor (Ashihara and Crozier, 1999). It also undergoes a slow catabolic process to give theophylline (1,7-dimethylxanthine) and other products (Figure 2). The rate of caffeine catabolism and biosynthesis depends on the species and age. The amount of caffeine in the coffee tree varies with the species, cultivars, stage of growth and environmental conditions such as soil fertility (Ashihara *et al.,* 1996). Studies have shown that plants growing in nitrogen-rich soils are able to synthesize more alkaloids than the same plants growing in nitrogen-poor soils (Gartland *et al.*,1980).



Caffeine



Theobromine



Theophylline

Figure 2. Some of the purine alkaloids occurring in coffee.

Caffeine has been found to be active against fungi such as *Collettotrichum Kahawae* and *Collettotrichum Glocosporiodes* (Buchanan *et al.*, 1981, Biratu, 1997). Among the three purines tested (Figure 2), caffeine was the most active. Complete *in vitro* inhibition of mycellial growth of the fungus *Collettotrichum Kahawae* was attained at 0.5% caffeine, comparable to 0.15% of the synthetic fungicide prochloraz (appendix B). Beans of coffee berry disease (CBD) resistant coffee selections in Ethiopia and cultivars in Kenya were found to be higher in caffeine content than the susceptible ones (Biratu, 1997), suggesting that caffeine is synthesized by the coffee tree for defense purposes.

Caffeine is a well-known alkaloid with a strong bitter taste. This taste, combined with the astringent taste associated with polyphenolic compounds has a depressive effect on voluntary feed intake by animals.

Ruminants and rats fed on coffee pulp show increased motor activity, which results in increased use of energy and ultimately a decrease in weight gain and food conversion efficiency. Caffeine is not the only substance causing these effects; chlorogenic acids have been shown to act in a similar way (Murillo, 1974). Therefore an additive or synergistic effect cannot be ruled out (Clifford and Ramirez-Martinez, 1991)

Caffeine also has a diuretic effect, particularly on ruminants. It causes increased urine output, which implies an increased loss of nitrogen, which if not compensated can easily deplete the animal of protein. This, in part, explains the emaciated state reported in animals fed on very high levels of coffee pulp (Madden, 1974). It may also explain the results of studies in which high levels of protein in the diet increased tolerance to higher

concentrations of coffee pulp (Flores, 1973). The protein supplementation may well compensate for the losses of nitrogen caused by higher urine excretion.

2.2.2. **Polyphenols.**

Polyphenolic compounds are a large, heterogeneous class of secondary plant metabolites. They occur in the plant as glycocides except the condensed tannins. There are three major classes of polyphenols occurring in fibrous feedstuffs (George and Hans-Joakim, 1989). These are;

(i) **Simple Polyphenolics.**

This class consists of monomeric compounds with multiple hydroxyl groups. Examples include gallic acid, egallic acid and chlorogenic acids. Chlorogenic acids is a term referring to the various isomers of caffeic, coumaric and ferulic acid esters of quinic acid that produce a green colouration on alkaline oxidation (Clifford, 1991). Figure 3 shows the structures of some of the simple polyphenols.

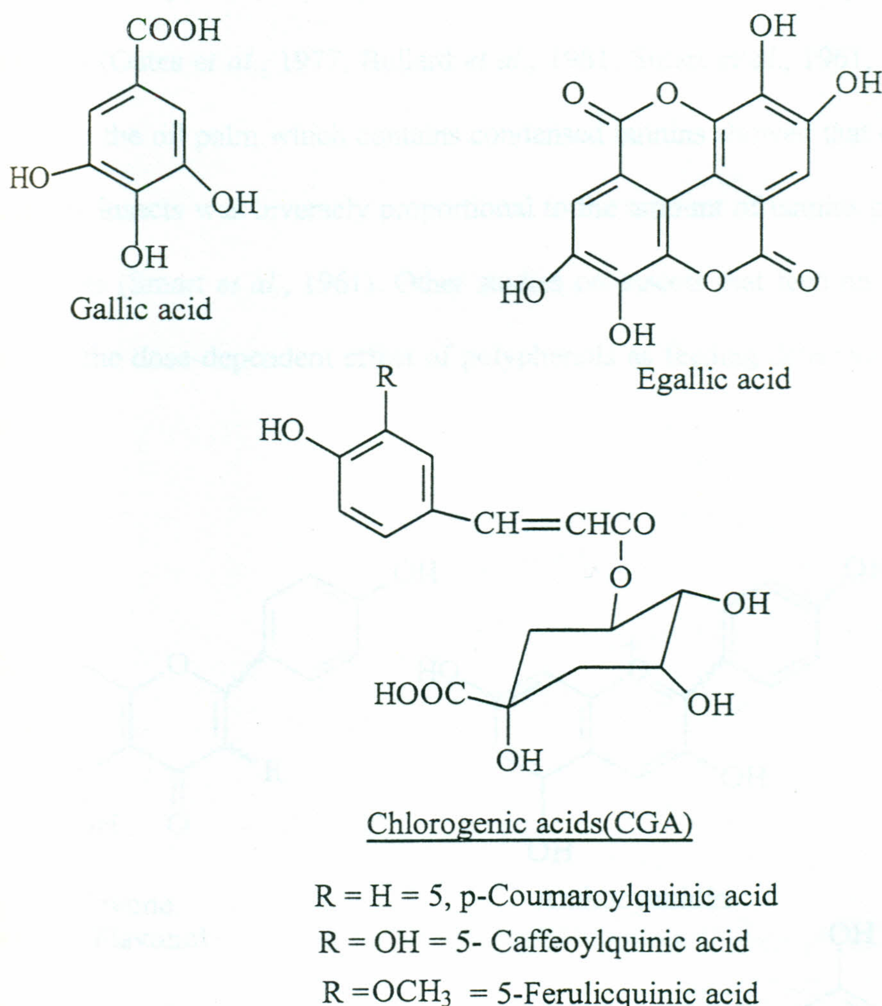
(ii) **Flavonoids.**

This is a class of secondary plant metabolites known for their ability to impart colour and flavour to plants. Flavonoids of forages can be assigned to four catecories; flavones, flavonols, catechins, anthocyanidins and isoflavones (Figure 4).

(iii) **Tannins.**

Tannin is an ill-defined group of polyphenolic compounds. Singleton and Kratzer (1973) defined tannins as polyphenolic compounds with a molecular weight greater than 500 that can precipitate proteins from aqueous solutions. There are two main classes of tannins; the hydrolisable tannins which are based on phenolic acids such as gallic acid (gallotannins) or ellagic acid (ellagitannins) substituting a sugar such as glucose, and the

condensed or procyanidin tannins based on C-15 repeating units of catechins, or related flavonol structure (Figure 5).



R = H = 5, p-Coumaroylquinic acid
R = OH = 5- Caffeoylquinic acid
R = OCH_3 = 5-Ferulicquinic acid

Figure 3. Examples of some simple phenolics.

Chlorogenic acids and both hydrolysable and condensed tannins have been isolated and characterized in coffee pulp (Clifford and Ramirez-Martinez, 1991). Qualitative spot tests for gallotannins failed, implying that coffee pulp contains mainly the egallotanins. Fresh coffee pulp has a deep red colour attributed to the polyphenols of low molecular weight, mainly the flavonoids. Upon contact with air, the pulp turns into a black hue. This change in colour has been attributed to enzymatic oxidation of the polyphenols to quinones,

which in turn combine with free amino acids and proteins to give dark complexes (Bressani, 1979).

Plants synthesize polyphenols as a defence mechanism against herbivores and pathogens (Oates *et al.*, 1977, Bullard *et al.*, 1981, Smart *et al.*, 1961, Ariga *et al.*, 1981). Studies on the oil palm which contains condensed tannins showed that damage to the plant caused by insects was inversely proportional to the amount of tannins present in the leaves of the trees (Smart *et al.*, 1961). Other studies on insects that feed on oak folliage further confirms the dose-dependent effect of polyphenols as feeding deterrants (Jung and Fahley, 1986).

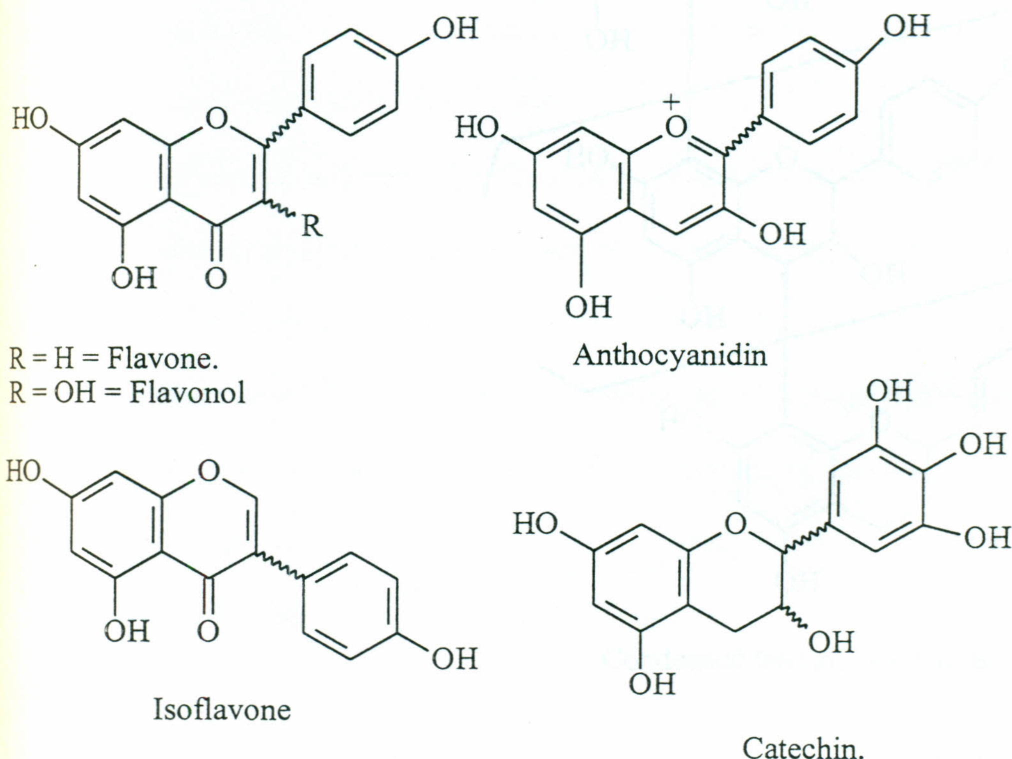
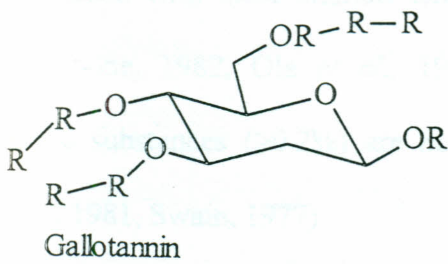
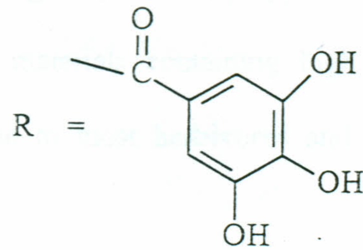
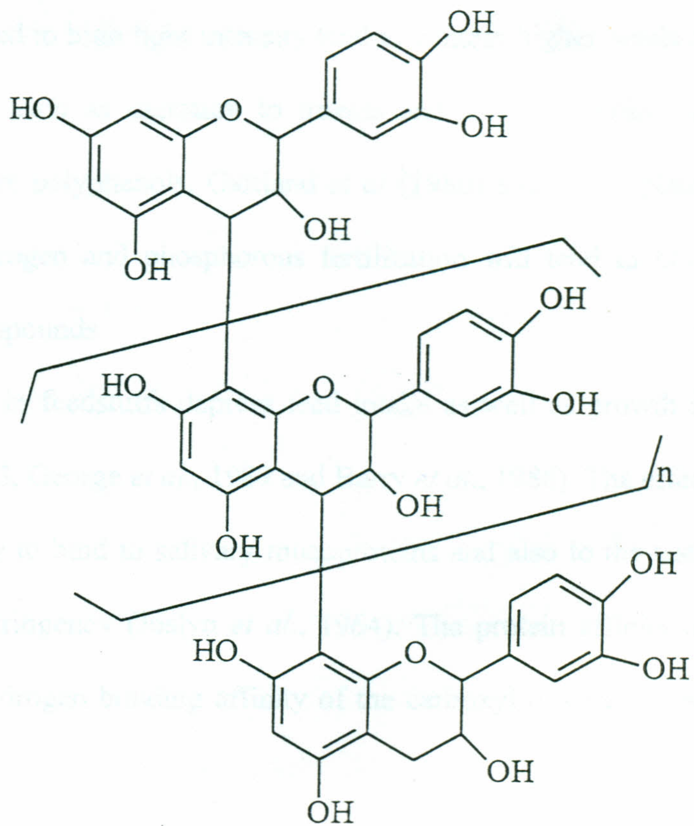


Figure 4. Examples of some common forage flavonoids.







Condensed tannin, n = 5 or 6

Fgure 5. Examples of tannins occurring in forages.

Polyphenols in plants have also been found to deter herbivores and birds, and plants with close association with these animals tend to have higher levels of polyphenols in their tissues (Harbone, 1982, Ola *et al.*, 1987). Plant materials containing high levels of polyphenolic substances (>0.2%) are not acceptable to most herbivores and pathogens (Ariga *et al*, 1981, Swain, 1977).

Plants have varying amounts of polyphenols depending on genetic factors, light intensity, and the absence or presence of environmentally induced stress (Del moral, 1972, Gartland *et al.*, 1980). Plants exposed to high light intensity tend to contain higher levels of polyphenols. Environmental stress such as exposure to insects and soil conditions can stimulate the plants to produce more polyphenols. Gartland *et al* (1980) found that plants growing in soils with minimal nitrogen and phosphorous fertilization will tend to have higher amounts of polyphenolic compounds.

High levels of polyphenols in feedstuffs depress feed intake as well as growth in animals (Singleton and Kratzer, 1973, George *et al.*, 1989 and Barry *et al.*, 1986). The effect on palatability is due to their ability to bind to salivary mucoproteins and also to the taste receptors in the mouth, causing astringency (Joslyn *et al.*, 1964). The protein affinity of polyphenols is due to the strong hydrogen bonding affinity of the carboxyl oxygen of the peptide bond (Rusell *et al.*, 1968).

Growth depression results from the complexation of polyphenols with dietary proteins leading to low-protein digestibility and increased faecal nitrogen concentration. Growth depression has also been attributed to the ability of polyphenols to complex with digestive enzymes involved in microbial fermentation of forages such as pectinases, cellulases, amylases and proteases, thus interfering with normal digestion (George *et al.*,

1989). The effect of tannins and other simpler phenolics on dry matter digestibility has been extensively studied. Total phenolics have been shown to be negatively correlated with *in vitro* forage digestion by ruminal microorganisms from cattle and removal of polyphenols by polyvinyl pyrollidone (PVP) treatment of extracts of alfalfa improved cellulose and protein fermentation (Jung and Fahley, 1986). Chlorogenic acids have also been shown to inhibit the action of ruminal microorganisms (Bitter, 1984). Feeding high tannin varieties of the lotus fodder to sheep resulted in dry matter disappearance depression *in vivo* (Cope and Burns, 1971), and addition of polyvinyl pyrollidone to lotus diets improved rumen and total fibre fermentation significantly (Barry *et al.*, 1986). This could be due to the tannin binding to the enzymes thus deactivating them.

Vohra *et al* (1967) reported that 0.5% tannic acid in feeds fed to chicks caused growth depression while a level of 5% caused a 70% mortality. In another study, 4% of tannic acid in diet caused severe growth depression in rats and 8% caused a 90% mortality (Glick and Joslyn, 1970). On the average a level of 1-2% of tannins in feedstuffs causes growth depression, while a value of 5% and above may cause death depending on the body weight and type of the animal (George *et al.*, 1989).

2.3. Treatment of Coffee Pulp.

Toxic compounds present in materials that are potentially good sources of nutrients can be eliminated by physical methods such as dry and wet heat dehydration or chemical methods such as extraction with organic solvents and maceration with acids and alkalis. For instance, cooking corn with lime and water makes the nutrients more available to humans and other monogastric animals (Bressani *et al.*, 1961). Efforts made towards preservation of

the pulp, elimination of the antiphysiological compounds and eventually making the pulp more acceptable to the animals include;

(i) **Drying.**

Coffee pulp contains a high moisture content (≈ 80%) and degrades very fast evolving unpleasant smells. This presents handling problems. An economically viable drying process must be considered for the pulp. This will help preserve the pulp and minimize the costs of transporting the material from the coffee-processing factory to the place of use.

Several drying technologies have been reported. These include sun drying, countercurrent tunnel dryers, belt dryers and rotary dryers. Sun drying is not particularly attractive because it is dependent on weather fluctuations but it is however, cheaper. Under poor weather conditions, the drying period is increased and this increases the chances of microbial degradation of the pulp. The disadvantage with the other methods is that their operational costs are high especially with regard to energy consumption (Molina, 1979).

Drying does not improve the quality of the pulp in terms of lowering the levels of the antiphysiological substances. They help to preserve the pulp and minimize handling and transport costs.

(ii) **Base/Alkali Treatments.**

Calcium and sodium hydroxide, sodium metasulphite, water and the combination of these compounds with physical treatments have been used in efforts to make coffee pulp more acceptable to the animals. Calcium hydroxide treatment has been extensively studied because of its numerous advantages. It is cheap, available and nontoxic to animals and

humans. In many cases it develops desirable organoleptic characteristics in the food, and at the same time a source of calcium (Brenes, 1979).

Although chemical treatments do not affect the levels of antinutritional factors in the pulp, the bases help to raise the pH and impart a characteristic taste, improving the voluntary intake of the pulp by the animals.

(iv) **Ensiling.**

Ensiling is basically fermentation under anaerobic conditions i.e. in the absence of oxygen. Thus the material must be sealed in a container to prevent re-entry and circulation of air during storage. The oxygen trapped in the herbage is rapidly consumed by respiratory enzymes in the plant. In the presence of oxygen, aerobic fermentation occurs and the material decays into a useless, inedible and frequently toxic product (MacDonald *et al.*, 1991).

In this method, the pulp is covered with an airtight material for anaerobic fermentation to occur. Under these conditions, anaerobic bacteria, mostly lactic acid bacteria break down sugars to give lactic acid, ethanol, acetic acid, water and carbon dioxide. Nitrogenous compounds are rarely changed because lactic acid bacteria have limited ability to ferment amino acids and other nitrogen containing compounds. It is thought that only two amino acids are extensively attacked, namely serine and arginine (Sneath *et al.*, 1986).

The types of silos in which a farmer may choose to ferment his crop are many and varied but the principle is the same. A typical silo consists of three slid walls some 2-3m in height and is often built under the ground to protect the silage from the weather. The crop is generally ensiled in a series of wedges, which are laid one upon the other progressively

along the silo and the top is sealed with plastic sheeting. Finally the top is weighted with some suitable material to prevent re-entry of air (Raymond *et a.,l* 1972). Silos for laboratory testing have been developed. These include test tubes, milk bottles, glass jars, sealed polyethylene bags and glass containers.

Ensiling has numerous advantages. Firstly, it is cheap compared to the other methods such as chemical treatments. Secondly, it reduces the levels of the antiphysiological substances significantly and thus improves the quality of the feed. Thirdly, it helps preserve the pulp; silage of coffee pulp can be preserved up to 18 months (Bressani, 1979). This is especially important because of the seasonal distribution of the coffee berry harvesting. Alot of pulp is produced over a short period of time and therefore there is need to preserve the pulp so that it can be available even during off-season.

Bressani (1979) has studied the fermentation characteristics of coffee pulp in which he experimented with coffee pulp alone and found that the levels of antinutritional substances are decreased significantly; total tannins decreased by 19% and caffeine by 33%. He then experimented with coffee pulp mixed with nappier grass and coffee pulp mixed with corn fodder and reported better results. Supplemental levels of other forages in the pulp silage tend to dilute the levels of caffeine and tannins.

2.4. Toxic substances in coffee pulp.

2.4.1. Pesticides residues.

The intensive use of copper-based fungicides and other organic based insecticides and fungicides in the coffee industry means that there is every possibility of finding pesticide residues in the coffee products especially the pulp. The pesticides commonly used for the control of pests and diseases in coffee fall in two categories; the organochlorine and the organophophorous pesticides.

The organochlorine pesticides are generally chemically stable. Their chemical stability arises from the C-C, C-H and C-Cl bonds, which are rather inactive under ordinary environmental conditions. They are also highly lipophillic. The two properties are responsible for the fact that organochlorines such as DDT tend to be persistent in the environment for a long time and are also capable of bioaccumulation through food chains. This has been a major concern to environmentalists. For this reason most organochlorine pesticides such as DDT and lindane have been banned from use in the market and others are progressively being replaced by other classes of pesticides.

All organochlorine pesticides are neurotoxic substances acting on the pheripheral nervous system as well as the central nervous system of the target pests. Among the organochlorines, DDT has been extensively studied. It has been shown that its insecticidal properties arise from its ability to bind to the proteins in the nerve membrane, thereby disturbing the sodium-potassium balance. This causes hyperactivity of nerves and finally death (Patlak and Horn, 1982). Other organochlorines act by similar mechanisms.

The history of organophosphorous pesticides dates back to the Second World War when German scientists embarked on a research of organophosphorous compounds with an

intention of using them in chemical warfare. After the war, research was directed to the development of pesticides. Since then they have overtaken the organochlorines in terms of annual global production and sales (Hassall, 1990). Owing to the presence of ester functional groups, the organophosphates such as malathion, fenitrothion and parathion offer fundamental advantages compared to the organochlorines. They can easily be degraded hydrolytically, enzymatically and biologically. They are more lethal to the pests and are applied in the fields at very low quantities. This reduces the risk of undesirable residues in the field as well as in the harvested crop (Schmidt and Fest, 1983). The organophosphorous compounds, though not persistent, are potentially dangerous because some of them are extremely poisonous and also because the effects of exposure are not felt immediately but are insidious in the onset. Their high toxicity is however partly offset by the fact that residues disappear rapidly from the surface of vegetation (Myria *et al.,* 1981).

The pesticides; chlorothalonil and triadimefon, and the insecticides; fenitrothion and chlorpyrifos are commonly used in the Kenyan coffee industry. The structures of some common pesticides used in the Kenyan coffee industry are given in appendix B. The literature on their use and toxicology is presented below.

(i). **Chlorothalonil** (2,4,5,6-tetrachloroisophthalonitrile).

This is a contact fungicide used to control fungal diseases in plants in nurseries, plantations and greenhouses. It has also been used in water purification since it has a wide range of algicidal action (Goulding, 1971). It persists on the surface of plant foliage and acts on the enzyme systems in fungi. Its fungicidal activity is presumably caused by the high reactivity of the chlorine atoms, whereby the compound reacts immediately with the thiol groups in the enzyme of the fungi (Vincent and Sisler, 1968, Tillman *et al.*, 1973).

Chlorothalonil is chemically stable in both acid and alkaline media. It is slowly degraded in the presence of light and has a long lasting action as foliage fungicide. It is reactive in soil but usually not absorbed from soil by plants. It is moderately persistent in the soil, with a half-life of up to three months in moderately moist soils. Under drier conditions the rate of degradation is slower. The main degradation product of chlorothalonil in the soil is 4-hydroxy-2,5,6-trichloroisophhthalonitrile. Some plants contain non-enzymatic catalysts that catalyze the hydrolysis of chlorothalonil into the hydroxy metabolite.

Chlorothalonil is a broad-spectrum fungicide used to control a wide range of fungal diseases. For this reason it is thought to be toxic to non-target fungi and bacteria in the soil. It has been found to be highly toxic to fish and other aquatic invertebrate animals. The 4-hydroxy metabolite is highly toxic to aquatic animals. It has however been found to be virtually non-toxic to birds and mammals. The acute toxic levels in some aquatic and terrestrial animals are given in the Table 5 below.

Humans exposed to chlorothalonil during spraying have developed eye irritation and skin irritation after 72 hours of exposure. However, no chronic effects have been reported. A level of 0.2mg/L inhaled or 0.5mg/L taken orally is considered harmful to man. The maximum residue level for chlorothalonil in or on animal feed (barley straw and fodder) is 20mg/kg (FAO/WHO, 1998).

Table 5. **Acute toxic levels of chlorothalonil for in aquatic and terrestial animals.** (U.S Department of Agriculture, 1994a).

Species	LC_{50}	LD_{50}
Trout	49μg/g	-
Water flea	70μg/g	-
Mallard	-	>4.640 g/kg
Bee	-	>181 μg/bee
Rat	-	>10 mg/kg

Key

LC_{50}- the concentration in air, water or food which will kill approximately 50% of the subjects.

LD_{50}- The dose which will kill approximately 50% of the subjects.

(ii). **Fenitrothion** (O,O-dimethyl O-(3-methyl-4-nitrophenyl) phosphorothioate).

Fenitrothion is a contact organophosphorous insecticide and a selective acaricide of low ovicidal properties (Spencer, 1981). It is effective against a wide range of pests including coffee leaf miners, locusts, rice stem borers, wheat bugs, Fluor beetles, grain beetles and grain weevils. It may also be used as a spray against flies, mosquitoes and cockroaches (U.S Department of Agriculture, 1998a).

Fenitrothion is a non-systemic and non-persistent insecticide. A half-life of approximately 50 hours in aerated river waters and 30-40 hours in the non-aerated waters has been reported (Sumitomo, 1977). In the soils, half-lives of 12-28 days have been reported depending on the type of soil. The major decomposition products are 3-methyl-4-nitrophenol and carbon dioxide. Fenitrothion is stable in sterilized soils, suggesting that microorganisms might play a major role in the decomposition, fungi being likely to be more

active than bacteria. The approximate residual period of fenitrothion on plant surfaces and other inert surfaces is 1-3 weeks depending on weather conditions (Harding, 1979).

Fenitrothion is oxidised by mono-oxygenase enzymes in animals, insects and plants and is changed to derivatives containing the P=O group, which are more powerful inhibitors of cholinesterase than was the original thiophosphate. After that, further degradation occurs by rapture of the P-OCH_3 linkage, which is more quickly metabolised than the P-O-phenyl linkage rapture occurring with parathion, a compound of similar structure.

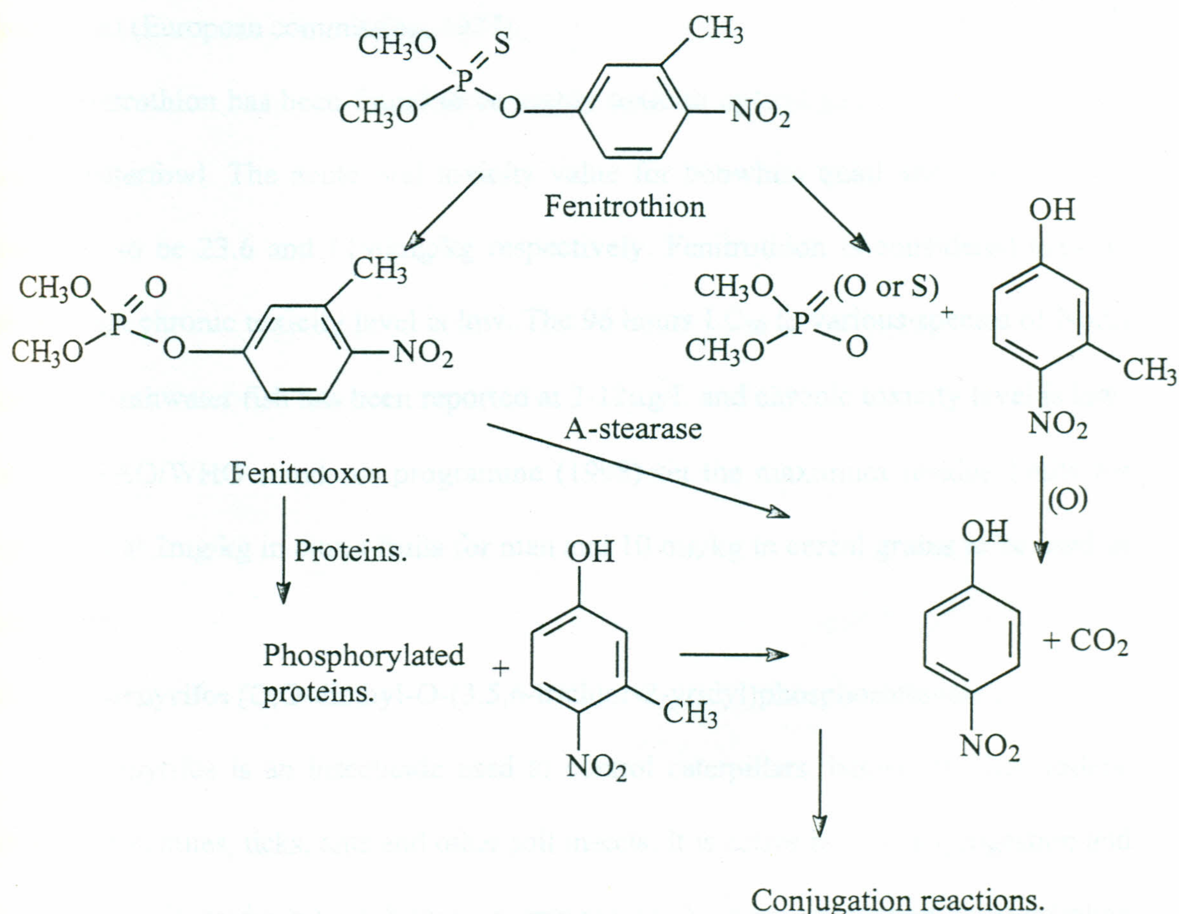


Figure 6. Degradation pathway for fenitrothion in animal and plant tissues (Saltatos and Minor, 1986).

This could contribute to fenitrothion's low mammalian toxicity since the cleavage of the P-OCH_3 linkage has been shown to be a detoxification mechanism for the organophosphates in animals (Hayes and Laws, 1990). Figure 6 gives degradation pattern for fenitrothion in animal and plant tissues.

Studies in the mouse, rat and quinea pig have shown that fenitrothion is rapidly absorbed from the mammalian gastrointestinal tract. The presence of the oxygen analogue has been detected in blood a minute after an intravenous injection of fenitrothion was administered (European commission, 1977).

Fenitrothion has been found to be highly toxic to upland game birds and slightly toxic to waterfowl. The acute oral toxicity value for bobwhite quail and mallards was determined to be 23.6 and 1190mg/kg respectively. Fenitrothion is considered toxic to fish although chronic toxicity level is low. The 96 hours LC_{50} to various species of North American freshwater fish has been reported at 2-12µg/L and chronic toxicity level is low. The joint FAO/WHO standards programme (1998) set the maximum residue limits for fenitrothion at 2mg/kg in citrus fruits for man and 10 mg/kg in cereal grains to be used as animal feed.

(iii). **Chlorpyrifos** (O,O-diethyl-O-(3,5,6-trichlor-2-yridyl)phosphorothioate).

Chlorpyrifos is an insecticide used to control caterpillars, bagworms, leaf rollers, borers, beetles, mites, ticks, ants and other soil insects. It is active by contact, ingestion and vapour action. It works by inhibiting an enzyme of the nervous system (acetylcholine esterase); this causes convulsions, paralysis and eventually death.

Chlorpyrifos is considered moderately persistent in the environment. It is strongly adsorbed by most soils and is relatively immobilized. Its half-life is in the range of 11-141

days in a variety of soils have been reported (Getzin, 1985). The main breakdown products in the soil, plants and animals are 3,5,6-trichloropyridinol (TCP) which is very persistent in soil but relatively non-toxic. For this reason it is, it is safe to use only one spray per season

Concentrations as low as 1.2µg/mL of chlorpyrifos in water are toxic to aquatic algae. Other aquatic plants may be adversely affected by exposure to levels expected from mosquito larvicide use of chlorpyrifos. Aquatic animals are affected by small levels of chlorpyrifos in the water; direct applications to water at rates as low as 0.01 pounds per acre may cause death to fish. Chlorpyrifos has been shown to bioaccumulate in fish tissues just like other organochlorine pesticides. The acute toxic levels to various species are shown in Table 6 below.

Table 6. The toxicological levels for chlorpyrifos in various species

(U.S Department of Agriculture, 1994b).

Species	LC_{50}	LD_{50}
Cold water fish	3.0ng/mL	-
Water flea	1.70ng/mL	-
Shrimp	0.035-0.11ng/mL	-
Birds	136-423ng/g	8.4-12mg/kg
Bee	-	0.114µg/bee
Rats	-	137mg/kg

The acceptable (maximum residue level) for man is 0.3mg/kg in citrus fruits and 1mg/kg in alfalfa fodder for animals (FAO/WHO, 1998).

(iv) **Triadimefon** (1-(4-chlorophenoxy)-3,3-dimethyl-1-(1H,-1,2,4-trizol-1-yl)-2-butanone).

It is a systemic fungicide belonging to the triazole family of organic compounds. It is used to control powdery mildews, leaf rusts and other fungal pests in cereals, fruits, vegetables and trees. It is mainly for folliar application and partly acts in the vapour phase within the plant. It also controls a number of bacteria including the species of rhizobium and pseudomonas (Oros and Gasztonyi, 1986).

Triadimefon is stable in water but is broken down by soil microorganisms easily. It has a half-life of six days in loamy soils (EPA, 1990). In humans and animals most of the compound was eliminated unchanged in the urine and faeces an hour after an oral dose. In higher plants and fungi, triadimefon is converted to triadimenol, which is even more toxic to fungi than the parent molecule. Further metabolism leads to the formation of chlorophenol and other conjugated derivatives. Figure 7 shows the metabolic pathway for triadimefon in animal and plant tissues.

Triadimefon, like other triazole fungicides acts by interfering with sterol synthesis in the organism by preventing the C-14 demethylation of the porphyrin system of cytochrome P_{450}. The nitrogen atoms in the triazole group binds to the porphyrin system, thus blocking the uptake of oxygen. The disruption of sterol biosynthesis leads to defective nerve membranes and eventually death (Sancholle *et al.*, 1984).

Triadimefon is not toxic to most species. However, it may cause certain growth disturbances when used above the effective doses. It is practically non-toxic to birds. The compound is slightly toxic to fish indicating that fish are more susceptible to the presence of the compound than are birds. It is a moderate poison for warm-blooded animals. When

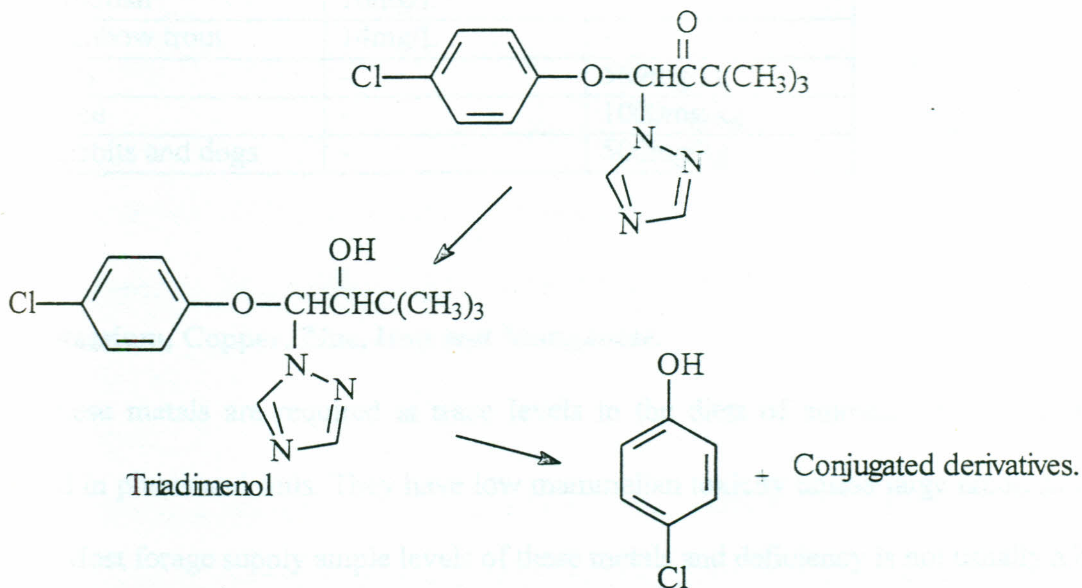


Figure 7. Metabolic pathway for triadimefon breakdown (Rouchard *et al.*,1982*)*.

absorbed through the skin or inhaled, it has very low toxicity and does not irritate the mucous membrane (Frohberger, 1973). The acute toxicity levels are shown in Table 7 below.

The maximum residue level for triadimefon in or on barley straw or fodder as recommended by FAO/WHO (1998) is 2mg/kg and that of its immediate metabolite, triadimenol has been set at 5mg/kg.

Table 7. **The acute toxic levels for Triadimefon to different species** (U.S Department of Agriculture, 1998b).

Species	LC_{50}	LD_{50}
Mallard ducks	-	4000mg/kg
Goldfish	10mg/L	-
Rainbow trout	14mg/L	-
Rats	-	569mg/kg
Mice	-	1000mg/kg
Rabbits and dogs	-	500mg/kg

2.4.2. **Potassium, Copper, Zinc, Iron and Manganese.**

These metals are required at trace levels in the diets of animals and are widely distributed in plants and soils. They have low mammalian toxicity unless large amounts are ingested. Most forage supply ample levels of these metals and deficiency is not usually a big problem. The problem is that of toxicity when the tolerance levels are exceeded.

(i) **Potassium.**

Potassium plays an important part alongside with sodium, chloride and bicarbonate ions in the osmotic regulation of the body fluids and in the acid-base balance in the animal (MacDonald *et al.*, 1990). Animal feeds usually supply ample potassium and there is no need of adding potassium supplements to livestock rations. Cases of potassium deficiency are rare except in cases where the soil contains abnormally low levels of potassium. Australian soils have been reported to be potassium deficient and cases of potassium deficiency have been reported in animals especially in sheep. Symptoms of potassium deficiency include retarded growth, paralysis, weakness and tetany and eventually death (Agric. Research Coun., 1984)

Sodium ions also play a role in the absorption of sugars and amino acids in the digestive tract (small intestines). Potassium and magnesium ions tend to replace sodium ions in the cells. It is suspected that the presence in a ration of high amounts of potassium will cause an increased replacement of cellular sodium ions by potassium ions, and therefore a greater excretion of sodium. Herbivores living largely on forage plants that are generally high in potassium require more common salt than those that consume rations rather lower in this mineral (Frank, 1984). Considerable amounts of potassium will cause an increased excretion of sodium, and therefore a greater need for common salt (Frank, 1984).

(ii) **Copper.**

Copper is a microelement that increases leaf retention and flowering, maintains protein-carbohydrate balance, improves root development and increases metabolic activity in the coffee plant (Hay, 1984, Aduayi 1973). Its use in the coffee industry dates as far back as 1920 when it was used for its tonic effect and later for the control of coffee berry disease (CBD), bacterial blight of coffee (BBC) and thereafter for leaf rust (Griffiths, 1971). Due to prolonged use of copper, there have been fears of possible copper phytotoxicity. Excessive copper in coffee has been shown to cause leaf yellowing, stunted growth and poor root development (Nay and George, 1960, Aduayi 1973).

In Kenya, the prolonged use of copper-based fungicides has been reported to increase copper levels in the soil and coffee leaves significantly (Aduayi, 1973, Spencer, 1966, Thuo *et a*l., 1994). Wanjohi (1991) reported a range of 9-200μg/g of copper in soils from a Kiambu district and ranges of 49.77-200.50 and 31.36-63.84μg/g in the coffee leaves and beans respectively. More recent work by Thou *et al* (1994) showed that leaf copper

levels were higher in the trees sprayed with copper fungicides (115.2-265.7μg/g) than the controls (61.0μg/g). Although experiments on coffee seedlings had shown extractable levels of copper above 100μg/g in the soil and 7.6 in the leaves as toxic (Aduayi, 1973), no case of copper toxicity has been reported in coffee trees. This has been attributed to the presence of phosphates and organic matter in the soil, which tend to complex the copper, making it less available (Aduayi, 1973, Bingham, 1963). This phenomenon hampers the early onset of copper phytotoxicity.

Although the role of copper in iron metabolism is not fully understood, small traces, of copper must be provided in animal feed to make possible the formation of haemoglobin. It also has many other basic functions; as an integral part of metalloenzymes such as urinase and as an essential element in bone formation. (Maynard *et al.*,1979). Symptoms of copper deficiency include depigmentation of coloured hair and black wool, a marked decrease in the rate of wool growth, bone disorders, ataxia and anaemia. (Underwood, 1971).

Cattle require 5.8 μg/g of copper in the daily ration, but a level of 115 μg/g is toxic (Suttle and Price, 1976). Sheep are more susceptible to copper toxicity than any other farm animal. The interaction of copper and molybdenum in the presence of sulphur is complex. Copper deficiency in ruminants is manifested by the same syndrome as chronic molybdenum poisoning and vice versa (Bunch *et al*, 1965). In acute poisoning by large doses of copper, loss of appetite, Jaundice, excessive salivation, abdominal pain and diarrhoea are usual symptoms in cattle (Macdonald *et al.*, 1987).

Swine are more tolerant of high dietary copper than ruminants (Pond *et al*, 1984). Supplemental levels of copper much higher than those required for normal body functions (200-250 μg/g of diet) for swine have been shown to stimulate increased growth rate and

feed efficiency. Although the mechanism of activity is not fully understood, it has been postulated that copper suppresses undesirable bacteria and stimulates the growth of desirable bacteria (Miller *et al.*,1979). A significant time period is usually required for the development of symptoms of chronic toxicity in swine and supplementing with zinc and iron can easily eliminate the toxic effects.

(iii) **Iron.**

Most of the iron in animals exists in complex forms bound to protein, either as porphyrin or heme compounds. It is also a component of many enzymes. Iron is required at trace levels by animals, mainly for haemoglobin formation.

The actual requirement for each animal depends on age, sex, nutritional status and state of health. Pigs require 50-60μg/g in daily diet; Chicks require75-80μg/g in daily diets but egg-laying hens require more. Iron deficiency in animals is rare except in pigs fed with rations that are very high in copper to promote growth. This is because their extremely high growth results in a dillution effect on the total body iron stores unless iron is fed ofr injected. Deficiency symptoms include anaemia and loss of appetite.

(iv) **Manganese.**

Manganese is required in trace amounts in animals as an enzyme activator and resembles magnesium in activating a number of phosphate transferases and decarboxylases. It is essential for normal bond formation and is also a component of most metalloenzymes (Pond *et al*, 1984). Owing to its widespread occurrence in foodstuffs and forages, cases of manganese deficiency are rare. They have been reported only in animals fed on forages that grow in soils are extremely poor in this metal. Deficiency symptoms include retarded growth, skeletal abnormalities and reproductive failure (Miller *et al.*, 1979).

There is a wide margin of safety between the toxic dose of manganese and the normal levels in foods. Rats, poultry and calves show no ill effects when fed on diets containing 820-100 mg/kg, but pigs show retarded growth at 500 mg/kg. For cows 1000 mg/kg of manganese in diet appears to be the maximum tolerance level, whereas for poultry and swine, 2000 and 400 mg/kg respectively are the upper limits. (Mertz, 1986).

(V) **Zinc.**

Zinc is a constituent of most metalloenzymes in the animal body such as phosphatase, Ribonuclease etc. It is required for normal protein synthesis and metabolism and is a component of insulin. Zinc is also an activator of most several metalloenzymes (Pond *et al*, 1984).

Although most feeds supply ample amounts, cases of zinc deficiency have been reported. Symptoms of zinc deficiency include abnormal growth, depressed appetite, poor feed conversion and parakerosis. The requirement for most species is less than 50mg/kg of diet.

Animals have a high tolerance of zinc poisoning. However, excess zinc in the diet may depress food consumption and induce copper deficiency (Macdonald *et al.*, 1990). Symptoms of zinc poisoning include anaemia, growth failure and death. Zinc fed at 1 g/kg of diet shows no ill effects in pigs, but levels of 4-8 g/kg may produce depressed growth, stiffness and haemorrage. Levels of between 900-1700 mg/kg of diet have been shown to depress appetite in sheep but to have no effect on cows. Although the allowed limit is 50 mg/kg of diet levels of less than 600 mg/kg are tolerated by most domestic animals (National Research Council, 1980).

2.5. Scope of the study.

The Kenyan coffee industry produces over 350000 tons of clean coffee together with similar quantities of by-products annually. Most of these by-products end up in nearby streams causing serious pollution problems. To alleviate these problems, safer and profitable disposal methods need to be explored. Possible alternatives may include use of coffee pulp as animal feed or organic manure.

Most of the coffee in Kenya is produced in the Central and Eastern provinces. These areas have high population densities and grazing land is rarely available. Consequently, prices of animal feeds have been going up in response to the high demand. There is need to identify cheaper alternatives of animal feeds such as agricultural by-products. Coffee pulp is important candidate since it is very cheap and its use would assist alleviate pollution problems in the long term.

Coffee by-products are contaminated with pesticides and any attempt to convert coffee pulp into animal feed should take into account the negative effects of these substances. Thus it is necessary to determine the levels of toxic and antinutritional substances in coffee pulp. Once the levels have been determined, an economically viable method of removing them should be sought. One such method is ensiling.

2.6. Objectives of the study.

The broad objective of this study was to assess the suitability of Kenyan coffee pulp for use as animal feed. The specific objectives were,

(i) To determine the levels of caffeine, total tannins, potassium, copper, zinc, manganese, and iron and pesticide residues in Kenyan coffee pulp.

(ii) To investigate the effect of ensiling on the levels of caffeine,

total tannins and pesticide residues in the coffee pulp.

2.7. Analytical Methods.

2.7.1. Analysis of Metals: Atomic Absorption-Spectrophotometry (AAS).

Atomic absorption spectrometry is a powerful instrumental technique for the determination of heavy metals in liquid samples. It provides a total metal content of the sample. Since the work of Alan Walsh in 1950`s, the growth of analytical atomic absorption spectrophotometry has been phenomenal. It has been used in the determinations of many metals at concentrations that range from trace to macroquantities (APHA-AWWA, 1975).

Principle.

When electromagnetic radiation is incident onto a vapour of metallic atoms, the atoms will be receptive of light radiation of their own specific resonance wavelengths. Upon absorption of radiation, the atoms are transformed from a low energy state (groundstate) characterized by E_1 to a higher energy state (excited state) characterized by E_2. The transition $E_1 \longrightarrow E_2$ results from the absorption of radiation of frequency, ν given by;

$$\nu = \frac{E_2 - E_1}{h}$$

Where h is planck`s constant.

The excited atoms may revert back to the ground state by emitting radiation of the same frequency. The transitions are always stimulated by the absorption of radiation from an

external source. The measurement of the radiation absorbed in such a transition forms the basis of AAS.

Instrumentation.

The most important components of AAS are;

(i) **Radiation source.**

This should emit stable, intense radiation of the resonance wavelength of the element to be determined and should guarantee high signal to noise ratios. The radiation sources commonly used in AAS are the hollow cathode lamps consisting of the test element and the gaseous discharge lamps (arc lamps).

(ii) **Atomizers.**

These convert the metal ions in the liquid sample vapour. Air- acetylene and nitrous-acetylene flames are commonly used, although electrically heated graphite atomizers are used for special analytical work. Refractory compounds like aluminium require high temperatures for atomization and the nitrous – acetylene flame is appropriate.

(iii) **Nebulizers.**

This converts the liquid sample into small droplets before the sample enters the atomizer. This is achieved by use of a gas moving at high velocity.

(iv) **Monocromators.**

A monocromator selects a given absorption line from spectral lines emitted from the light source or background emission from the flame. The most common monocromaters in AAS are prisms and gratings Gratings have the advantage of constant

performance throughout the electromagnetic spectrum and are often used. However, prisms have higher performance in the ultraviolet region.

(v) **Detectors, Amplifiers and Read-out systems.**

The photomultiplier tube is used in most modern instruments. In the photomultiplier tube, there is an evacuated envelope, which contains a photocathode, a series of dynodes, which amplify the optical signals and an anode. A photon from the monocromator strikes the photocathode and dislodges an electron which is the accelerated to dynode one. The accelerated electron in turn ejects two or more electrons from dynode one. These electrons are accelerated to dynode two, three, etc resulting in the ejection of more and more electrons which eventually reach the anode as an amplified electron current. The amplified electron current from the photomultiplier tube is then fed to an electrical amplifier, which is then read out on an analogue or digital display. Most modern instruments are interfaced to a computer processor.

This method is generally free from spectral interference or radiation interference since each metal has its own characteristic wavelength (APHA-AWWA, 1975) and the source lamp is composed of the element being determined. Several workers have used AAS to determine various metals in water, soils and plant tissues with variable success (Mitei, 1996, Kimei, 1996, Munga, 1998, Maroko, 1989 *inter alia*). Detection limits vary with the metal and the specific instrument but it is generally less than 0.1μg/ml (Chatwal and Anand, 1991). The incorporation of appropriate computer software in modern Atomic Absorption Spectrophotometers has made AAS one of the most rapid analytical techniques. Thus in view of the high accuracy precision, selectivity, sensitivity and rapidity attainable in analysis by AAS, the method was adopted in this work.

Other spectroscopic methods that have been used in the analysis of metal ions include colorimetry (Allen, 1974), fluorimetry, mass spectrometry (Jenkins and Majev, 1967), X-ray spectroscopy (Boyer *et al.*, 1978), fluorescence absorption spectrophotometry, arc emission spectroscopy and inductively coupled plasma (Greenfelds *et al.*, 76). Electrochemical methods such as polarography and ion selective electrodes have also been applied in the determination of heavy metals in water and other materials (Burkharov *et al.*, 1968). Each analytical technique has its own strengths and weaknesses and the choice of a method will depend on the nature of sample to be analyzed and the availability of equipment and reagents.

2.7.2 . Analysis of Tannins.

Polyphenols have been neglected for a long time, partly because of their complexity and diversity. However, their nutritional effects on animals and the fact that they also have pharmacological properties has rejuvenated research in this group of compounds (George *et al.*, 1989). One of the challenges has been to develop methods of identification and quantification. Tannins have received special attention due to their profound effects on animals. The methods that have been developed for tannin determination are divided into three types; colourimetric, gravimetric and protein precipitation methods.

2.7.2.1. Colourimetric methods: Ultraviolet-Visible (UV) Spectrophotometry.

UV- Spectrophotometry is one of the most popular analytical techniques in the determination of molecular species. It involves the measurement of the amount of radiation absorbed by molecules or ions. It is known for its rapidity, high selectivity, a reasonably high detection limit and affordable instrumentation.

Principle

The total energy in a molecule is a sum of contributions from electronic, rotational and vibrational energies i.e

$$E_{tot} = E_{el} + E_{rot} + E_{vib}$$

The electronic energy is generally larger than the other two and electronic transitions ordinarily involve energies corresponding to UV and visible region of the electromagnetic spectrum. Thus molecular absorption in the UV-Visible region depends on the electronic structure of the molecule.

There are three distinct types f electrons involved in organic molecules. These are designated as Sigma (σ), Pi (π) and nonbonding (n) electrons depending on the molecular orbital they occupy. Energy absorbed in the UV-Visible region by molecules causes transitions of valence electrons in the molecule. These transitions are $\sigma \rightarrow \sigma^*$, $n \rightarrow \sigma^*$, $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$. The modern spectrophotometers operate in the range of 200 to 800nm.Thus the transitions $\pi \rightarrow \pi^*$ (ultraviolet) and $n \rightarrow \pi^*$ (near UV and visible) are the ones normally involved in UV-Visible spectroscopy measurements.

The absorption of radiation by molecules obeys Beer-Lambert law, which in the simplified mathematical form states;

$$A = \epsilon cl$$

Where, A is absorbance, c is concentration in moles per litre and l is the cell pathlength in centimetres. Since absorbance depends on the concentration of the absorbing species, it is possible to quantitatively determine the amount of a given species present.

Instrumentation.

The important components of a UV- Spectrophotometer are;

(i) **Radiation source.**

In UV spectrophotometers the most commonly used radiation sources are hydrogen, deuterium, xenon and mercury vapour lamps. When electrons are passed through these gases at low pressure, they produce a continuum emission, which covers the visible region and down to the UV region of the electromagnetic spectrum. Tungsten filament incandescent lamps are commonly used for the visible and near-IR regions.

(ii) **Monochrometers.**

Monocrometers for UV radiation consist of prisms of quartz, fused silica or echlette gratings as the disperssive element. The mirrors in the optical system are front surfaced because glass begins to absorb in the UV region

(iii) **Detectors.**

UV Spectrophotometers use either vacuum photoemission photubes or electron multiplier phototubes as detectors. The photocathode surfaces are coated with antimony, caesium, selenium or any other element with high sensitivity to UV radiation. The photocurrent output of these detectors is amplified and then recorded.

The colourimetric methods that have been used in the determination of polyphenolic compounds in plant samples are;

(a) **Folin-Dennis method.**

This method is based on the reduction of phenols by phosphomolybdic acid in aqueous alkali. It involves a mild oxidation of phenol groups to quinones. The result is a blue adduct which is then measured spectrophotometrically at 760nm. The method

determines the total free phenolic groups and is therefore a method for determination of total soluble phenolics, hydrolysable tannins and condensed tannins (Waterman and Moles, 1994).

The limitation with this method is that it does not differentiate between tannins and many phenolic compounds that are not tannins. It is also subject to interference from other compounds such as ascorbic acid, tyrosine.

(b) **Vanillin-HCl assay.**

This method is specific for condensed tannins. It involves an exo-type reaction in which vanillin (4-hydroxy-3-methoxybenzaldehyde) reacts with the meta-substituted A-ring of flavanols to form a chromophore which is then measured spectrophotometrically at 500nm. The number of flavanols produced is proportional to the absorbance of the solution.

The disadvantage with this method is that low molecular weight flavonols overreact and large polymers underreact. In addition, a catechin is used as a standard. This monomer gives the maximum optical density leading to underestimation of large polymers (Waterman and Moles, 1994).

(c) **Butanol-HCl assay.**

This is based on an endo-type reaction, which involves HCl catalyzed depolymerization of condensed tannins in butanol to yield a red anthocyanidin product that can be measured spectrophotometrically at 550nm. This method is specific for condensed tannins. The fact that tannin polymers are cleaved into dimers and trimers rather than monomers hence leading to underestimation is a major limitation of this method (Waterman and Moles, 1994).

(d) **Rhodanine assay.**

This method is specific for gallotannins. It involves acid hydrolysis of gallotannins to release gallic acid. The resultant gallic acid is then reacted with the dye rhodanine to produce an intense colour that is measured spectrophotometrically (Waterman and Moles, 1994).

(e) **Wilson and Hagerman assay.**

This method is specific for ellagitannins. It involves acid hydrolysis of ellagitannins to release ellagic acid. The resultant ellagic acid is then reacted with sodium to give a coloured solution that can be measured spectrophotometrically (Waterman and Moles, 1994).

2.7.2.2. Gravimetric methods.

(a) **Ytterbium method.**

It is based on the ability of trivalent ytterbium ion to selectively precipitate polyphenols from plant extracts. Quantitative information is then obtained by considering the mass loss due to precipitation. It determines soluble tannins in plant extracts; insoluble tannins are not measured.

The advantage with this method is that no standards are required. The precipitate can easily be dissolved in oxalic acid solution to yield a solution of polyphenols and an insoluble Yb-oxalate. The polyphenol solution can be used for further analysis such as colourimetric, chromatographic and inhibition studies. The disadvantage is that not all polyphenols are precipitated. Repeatability in plants with low levels of tannins has also been found to be low (Reed *et al.*, 1985).

(b) **Polyvinyl Pyrrolidone (PVP) method.**

This method makes use of the ability of polyvinyl pyrrolidones (PVP) to bind irreversibly to tannins. The amount of tannins is calculated from the mass of tannin-PVP precipitate. This method determines only soluble tannins in plant extracts. It is not very sensitive and tends to underestimate tannins (Makkar *et al.*, 1995)

2.7.2.3. **Protein Precipitation methods.**

These methods are more closely related to the biological effects of tannins. They make use of the formation of complexes between tannins and bovine serum albumin embedded in agar. Plant extracts are placed in a well in the agar. They diffuse in the agar and precipitate the albumin if tannins are present, forming an opaque circle. The diameter of the circle is proportional to the amount of tannins in the extract. By using appropriate standards, the amount of tannins can be determined (Hagerman, 1987).

The method determines only soluble tannins and is less useful for quantification than the colourimetric methods. The only advantage is that it requires limited laboratory facilities.

Due to the complexity and diversity of tannins, none of above methods is completely satisfactory. Among these classes of methods, colourimetric methods have better repeatability and are more useful for quantification. In this study, the Folin-Dennis method was used.

2.7.3. Analysis of Caffeine and Pesticide Residues.

(a) Gas Chromatography (GC).

Chromatography is a separation technique that is based on the difference in the rates at which the components of a mixture move through a stationary surface (liquid or solid) under the influence of a mobile phase (gas or liquid). Among the various separation techniques in chromatography, gas chromatography is the most pre-eminent. Since its introduction in the 1950s, it has grown to be the most popular method for the analysis of a wide range of volatile or easily volatilizable compounds. It combines the advantages of high selectivity, sensitivity, wide applicability and rapidity as well as simple and relatively inexpensive equipment (Skoog and West., 1982). It has been used in the analysis of pesticides, hydrocarbons and even some organometalics.

Gas chromatography is the most commonly used method for pesticide residue analysis (Keith, 1993). This is because most pesticides are easily volatilized within the temperature range normally used in gas chromatographs and the high sensitivity of the instrument. Detection limits vary with instrumental conditions, detectors and the nature of the sample.

There are several detectors available for detection in Gas Chromatography, the choice of which depends on the type sample to be analyzed. The detectors that were used in this work are flame ionization detector (FID) and electron capture detector (ECD). FID is the most popular detector used in Gas Chromatography. It is easy to use, gives a stable response and is sensitive to most organic compounds. It is based on the electrical conductivity of gases. The column effluent is mixed with hydrogen and passed into a chamber through which air is passed. The hydrogen is ignited to produce a continuous

flame. As compounds from the column enter the flame, they undergo combustion with a small proportion of the carbon atoms undergoing ionization. An electrode, which is polarized with respect to a jet in the combustion chamber collects these ions and the resulting electrical current is amplified to provide the chromatographic signal.

The electron capture detector is extremely sensitive to molecules containing highly electronegative atoms, such as halides, nitrates, nitriles, peroxides and even organometalics. It is therefore a popular detector for trace level determination of pesticides and halocarbon residues in environmental samples. It consists of an ionization chamber containing a radioactive source (nickel-63 or tritium, Ti^3H, Sc^3H) which emits beta-particles. During operation there is a constant flow of nitrogen or 5-10% methane in argon flowing through the cell, which is ionized by this emission causing the liberation of thermal electrons. These electrons are collected by a positively charged electrode, thus generating a small constant current, the standing current. An electrophilic species in the sample will capture the free thermal electrons, thus reducing the standing current. The output signal is derived by amplifying and inverting the resultant current (Keith, 1993).

In this work a Perkin-Elmer Sigma 300 gas chromatograph was used. Electron capture detector, tritium source was used for pesticide residue analysis, while a FID was used for caffeine analysis.

(b) **High Performance Liquid Chromatography (HPLC).**

High Performance (pressure) Liquid Chromatography (HPLC), is a modification of the classical liquid Chromatography. It employs high pressures to force the mobile phase (liquid) through columns that contain very small particle sizes (5-10μm) and can therefore attain high column efficiencies (Skoog and West, 1982). HPLC is applicable to

nonvolatile or thermally unstable compounds since it operates at room temperature or temperature close to room temperature. It is sensitive, highly selective, has wide applicability and can be readily adapted for quantitative analysis. Unlike in GC, there is no highly sensitive universal detector system available in HPLC. The most common detectors are ultraviolet and fluorimetric detectors. Sensitivity varies with nature of analyte and instrumental conditions, but detection is possible down to the nanogram level for both detectors (Skoog and West, 1982).

HPLC has been used extensively in the analysis of amino acids, some polyphenols such as catechins, caffeine and vitamins in plant and animal samples (Clifford, 1991, Liang *et al.*, 1990, Ramirez-Martinez, 1988 Owuor, 1986). It is the traditional method for caffeine analysis.

♦ CHAPTER THREE

3.0. EXPERIMENTAL

3.1.-Cleaning of glassware and plastic containers.

All glassware and plastic containers used in this work were first soaked in chromic acid then washed in detergent and finally rinsed with distilled water. When not in use, all containers for elemental analysis were soaked in 10% nitric acid solution. Immediately before use, they were rinsed in de-ionized water then dried in the oven at 110^{0}C for not less than two hours. Plastic containers were dried at room temperature. Glassware for pesticide residue analysis was rinsed in distilled water then dried in the furnace at 250^{0}C for one hour before use to avoid any contamination. The extraction thimbles for pesticide analysis were first soaked in acetone then dried in the oven at 50^{0}C for at least 30 minutes.

3.2. Standard stock solutions and Reagents.

A 1000 μg/ml stock solutions for each standard was prepared from their pure compounds/elements or their salts. All the solvents used were of analytical grade and the standards' percentage purity was at least 98.5%. From the stock solutions, the working standard solutions were obtained by carrying out appropriate dilutions. The procedures followed in the preparation of the respective standard stock solutions are given below:

(i) **Caffeine.**

90.2mg of anhydrous caffeine standard, obtained from BDH (98.7% pure), was weighed in a weighing boat, transferred quantitatively into a 100ml volumetric flask and then dissolved in ethyl acetate, obtained from Aldrich (density, 0.902g/ml). Once

dissolved, the volume was made to the mark. This solution contained 1000µg/ml caffeine.

(ii) **Tannic acid.**

1.0g tannic acid, obtained from Aldrich (98.5% pure), was weighed in a weighing boat and dissolved in 1.0 L of distilled water in a 1.0 L volumetric flask and then the volume was made to the mark to give a stock solution containing 1000µg/ml tannic acid.

(iii) **Pesticides.**

The weighing boats were first rinsed with acetone to remove any contaminants. 66.0mg each of chlorothalonil (99.8% pure), fenitrothion (98.5% pure), chlorpyrifos (99.4% pure) and triadimefon (99.6% pure) pesticide standards was weighed and transferred to 100ml volumetric flasks (all the pesticides were obtained from BDH), dissolved in n-hexane (Aldrich) (density 0.66g/ml) then the volume made to the mark. The solutions obtained contained 1000µg/ml of each of the pesticide standards.

(iv) **Copper.**

0.50g of copper foil was weighed and transferred into a conical flask. 5ml of conc. hydrochloric acid were added followed by 1ml of conc. nitric acid. After the reaction had stopped it was boiled to expel nitrogen dioxide gas then allowed to cool. The contents were transferred to a 500ml volumetric flask. The solution was finally made to 500ml with de-ionized water. The solution prepared contained 1000µg/ml of Cu^{2+} ions.

(v) **Zinc.**

0.501g of pure zinc metal was weighed and put in a 500ml volumetric flask containing about 100ml of de-ionized water and 5ml of conc. HCl, and allowed to react.

After all the zinc had been consumed, the content was diluted to the mark with de-ionized water. The resultant solution contained 1000µg/ml of Zn^{2+} ions.

(vi) **Iron.**

3.52g of ammonium ferrous sulphate hexahydrate, obtained from BDH (98.8% pure) was weighed and transferred into a 500ml volumetric flask and dissolved in about 100ml of de-ionized water. 5 ml of a 2M sulphuric acid solution was added to oxidize the Fe^{2+} ions. The contents were finally made to the mark with de-ionized water. The final solution contained 1000µg/ml Fe^{3+} ions.

(vii) **Manganese.**

1.44g of dry Potassium permanganate (BDH) crystals (99.2%, pure) was weighed and put into a pyrex conical flask, dissolved in about 100ml of de-ionized water and 10ml of conc. sulphuric acid added slowly. The permanganate ions were reduced to Mn^{+2} by dropwise addition of a 10% sodium metasulphite solution until the solution became colourless. 2ml of conc. nitric acid were added to oxidize the excess sulphuric acid. This solution was then cooled and transferred into a 500ml volumetric flask and finally made to the mark with de-ionized water. The resultant solution contained 1000µg/ml Mn^{2+} ions.

(viii) **Folin-Denis Reagent.**

This was prepared by refluxing 100g of sodium tungstate dihydrate, 20g Phosphomolybdic acid (both obtained from BDH) and 50ml of conc.orthophosphoric acid in about 750ml of water for 2 hours. The contents were then allowed to cool and diluted to one litre with distilled water.

3.3. **Sampling and sampling sites.**

Fresh coffee pulp samples were collected between September to December 1998 from a total of fifteen coffee processing factories located in Thika and Kiambu districts. The coffee growing areas in these districts are divided into three agro-ecological zones based on climate, soil fertility and altitude. The zones are; the coffee-tea zone or upper midland-1 **(UM-1)**, the main coffee zone or upper midland-2 **(UM-2)** and the marginal zone or upper midland-3 **(UM-3)**. Altitude increases from UM-3 to UM-1 and so does rainfall and soil fertility (Table 8).

Table 8. Mean annual climatic conditions for the three agro-ecological zones for the period 1977-1987 (Coffee Research Foundation, Agronomy section).

Agro-ecological zone	Altitude(m, above sea level)	Annual temperate (^oC)	Annual rainfall (mm)	General soil fertility group.
UM-1	1700-1820	18.7-18.0	1300-1600	high
UM-2	1580-1760	19.5-18.4	1000-1400	Moderate to high
UM-3	1520-1580	19.9-19.5	800-1200	moderate

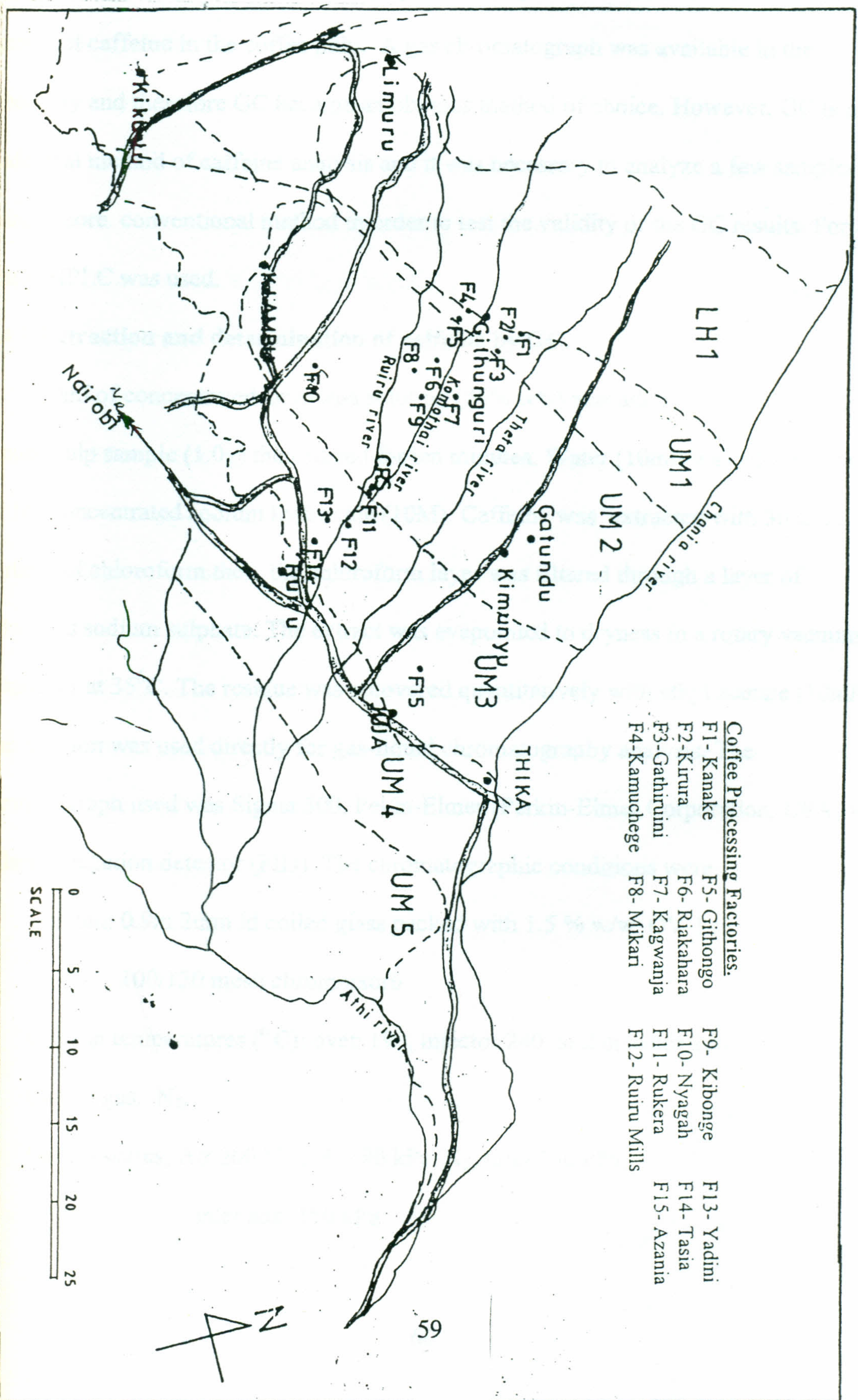
UM-2 and UM-1 are dominated by the co-operative sector. The sector comprises of small holder farms owned by individual farmers who have formed cooperative societies for the purposes of purchasing farm inputs, processing and marketing their coffee among others. Some of these farms are well managed and others are poorly managed. UM-3 is dominated by the estate sector. The estates are generally well managed, as they are private farms owned by big agro-business companies. All these farms grow arabica coffee.

Pulp samples were collected from five factories in each zone. UM-1 was represented by Kamuchege, Kirura, Kanake, Kathiruini and Githongo factories, UM-2 by Mikari, Kibonge, Kagwanja, Nyagah and Riakahara factories, and UM-3 by Azania,

Tassia, Yadini, Ruiru mills and Rukera factories. No regard was given to the actual source of the pulp samples. The results of this work therefore represent the mean caffeine levels in coffee pulp of the various cultivars of arabica coffee grown in these regions. Figure 8 shows the locations where the samples were collected.

About 1kg of fresh pulp was sampled from each factory. The samples for elemental analysis were collected in polyethylene bags, while those for pesticide residue analysis were first wrapped in aluminium foil before being put in polyethylene bags. They were then transported immediately to the laboratory and kept in a deep freezer awaiting analysis. Pesticide residues were extracted from the pulp samples within 48 hours of sampling. About 100g of each sample was first partially dried in the oven at 50°C for 3 hours to reduce the water content to within 50-60%, then tightly packed in polyethylene bags and sealed using a sealing machine. This was done in triplicate for each sample. The sealed samples were kept in the dark in closed glass containers.

Figure 8. A map showing the locations of the factories from where the pulp samples were collected.



3.4. **Determination of Caffeine.**

Both GC and HPLC chromatographic techniques were used to determine the amount of caffeine in the coffee pulp. A gas chromatograph was available in the laboratory and therefore GC became an obvious method of choice. However, GC is not a traditional method of caffeine analysis and it was necessary to analyze a few samples using a more conventional method in order to test the validity of the GC results. For this reason HPLC was used.

3.4.1. **Extraction and determination of caffeine by GC.**

1.0ml of concentrated ammonia solution (25% w/v) was added to the dried and ground pulp sample (1.0g) then stirred for ten minutes. Water (10ml) was added followed by 1ml concentrated sodium hydroxide (10M). Caffeine was extracted with 30 and 20ml portions of chloroform then the chloroform layer was filtered through a layer of anhydrous sodium sulphate. The extract was evaporated to dryness in a rotary vacuum evaporator at 35^0C. The residue was recovered quantitatively with ethyl acetate (10ml). This solution was used directly for gas-liquid chromatography analysis. The chromatograph used was Sigma 300, Pekin-Elmer (Perkin-Elmer Corporation, USA) with a flame ionization detector (FID). The chromatographic conditions were;

Column: 0.9m 2mm id coiled glass packed with 1.5 % w/w, OV-1 on
100/120 mesh chromorsorb.

Column temperatures (^{o} C): oven 190, injector 240, and detector 250.

Carrier gas, N_2.

Gas pressures; Air 200 kPa, H_2 180 kPa, N_2, inlet 350 kPa,
inlet aux. 180 kPa.

Injection volume; 3.0μL

Detector; Flame Ionization Detector (FID).

Intergrator; Perkin-Elmer, LCL-100.

3.4.2. **Extraction and determination of caffeine by HPLC.**

The samples were extracted as described in section 3.4.2. They were then evaporated to dryness in a rotary vacuum evaporator at 35^0C then recovered with distilled water. The final volume was made to 50ml. This sample was the one used for caffeine analysis by High Performance Liquid Chromatography (HPLC). The following were the HPLC chromatographic specifications;

Chromatograph; Shimadzu (Shimadzu Corporation, Japan).

Pump; shimadzu LC 6A.

System controller; Shimadzu SCL-6A.

Column; ODS.

Mobile phase; Isocratic, Methanol: Water: Acetic acid (40: 59.5 : 0.5).

Injection Volume; 2μL, loop injection.

Flow rate; 0.5ml/min.

Pressure; $112kgfcm^{-2}$

Detector; Ultraviolet Visible detector, SPD-10 AV Shimadzu at 280nm

Intergrator; Shimazdu C-R3A.

3.5. **Estimation of Total Tannins.**

Dried and ground coffee pulp (200mg) was weighed into a weighing boat and transferred into a 50 ml round bottomed flask and then extracted overnight (cold extraction) with 20ml of 70% acetone in distilled water. The pulp extract was then

filtered through a Whatman NO 40 filter paper, rinsed with acetone and then made to the 50ml mark with distilled water. A 1.0 ml aliquot of this extract was pipetted into a 100ml volumetric flask containing 75ml of distilled water. To this solution, 5ml of Folin-Denis reagent was added followed by 10ml of saturated sodium carbonate solution. It was then made to the mark with distilled water, mixed well, allowed to stand for 30 minutes and absorbance measured at 760nm (AOAC, 1995) using a ultraviolet-visible spectrophotometer, model, Perkin-Elmer Lambda 15, (Perkin-Elmer Corporation, USA). Quantification was done using a calibration curve of absorbance versus concentration for a tannic acid standard treated in the same way as the samples.

3.6. Analysis of pesticide residues.

3.6.1. Solvent systems and Recovery studies.

In this work a multiresidue analytical method for the extraction of both organochlorine and organophosphorous pesticides was followed. As a cost cutting measure, it was necessary to develop a multiresidue extraction method and look for a convenient solvent system for extraction. This was done as follows:

About 150g of fresh coffee pulp was weighed into a 500ml beaker then blended using a pestle and mortar that had previously been washed and rinsed in acetone. It was then divided into four 30g portions in 100 ml beakers and transferred into the extraction thimbles. Three of these samples were spiked with 125μL each of 1000μg/ml standard solutions of chlorothalonil, fenitrothion, chlorpyrifos and triadimefon pesticides and allowed to homogenize for one hour. One of the samples was not fortified with pesticides. They were then placed in the soxhlet apparatus and extracted simultaneously using different solvent systems. These were 100% toluene, toluene-hexane (3:1), toluene-

hexane (1:1), 100% diethyl ether, diethyl ether- hexane (3:1) and diethyl ether-hexane (1:1). The extracts were then reduced to a volume of about 2ml, eluted through the fuorisil column and then analyzed as described in Section 3.6.2 below.

3.6.2. Extraction and analysis of pesticide residues.

(i) **Extraction.**

The blended coffee pulp sample (30g) was put in the extraction thimble and extracted with diethyl ether- n-hexane mixture (3:1) for 4hours, at the rate of three extraction cycles per hour, using the soxhlet extraction apparatus. The extract was reduced to about 2ml using the rotary vacuum evaporator at 35^0C.

(ii) **Clean up.**

Fluorisil, activated by heating at 600^0C for one hour in a muffle furnace and stored in the dessicator for not more than five days was used. A column (id 20mm, length100cm) was filled with fluorisil (15g) under a 4cm layer of anhydrous sodium sulphate and the sample quantitatively transferred to the column. It was then eluted with 150ml of diethyl ether-acetone mixture (49:1). The eluate was reduced to about 2ml using the rotary vacuum evaporator at 35°C, transferred quantitatively into a 25ml volumetric flask and then made to the mark with n-hexane.

(iii) **Analysis.**

The solution obtained from the clean up above was used directly for gas chromatographic. Calibration curves of peak areas versus concentration for each pesticide standard was prepared. These were used to calculate the concentrations of the pesticide residues in the pulp extracts. The chromatogram used was Sigma 300 with the following specifications;

Column; 1.8m, 2mm id coiled glass packed with 3% w/w, OV-1

on 80/100 mesh chromorsorb.

Working temperatures (° C); Oven 190, injector 240, and detector 300.

Gases; carrier gas N_2, make up gas, N_2.

Gas pressures; inlet 360 kPa, inlet aux. 320 kPa.

Flow rate; $50 ml min^{-1}$.

Injection volume; 3.0μL.

Detector: Electron capture, tritium (^3H) source.

Intergrator; Perkin-Elmer, LCL-100.

3.7. Metal Analysis.

Dried Coffee pulp sample (0.5g) was ashed overnight at 450°C in a muffle furnace then digested with 2ml of a mixture of conc.$HClO_4$ and HNO_3 (3:1). 10ml of 0.5M HCl was added followed by 1 ml of freshly prepared 5% $NaNO_2$ solution. After boiling gently for 5 minutes, the contents were transferred to 25 ml excelo tubes and made to the mark with de-ionized water, filtered through a whatman filter paper, No.40 into a polyethylene specimen tube. The trace metals were determined by Atomic Absorption Spectrophotometer (AAS), model SP9 (Perkin-Elmer corporation, USA) at their respective wavelengths while potassium were determined by Atomic Emission Spectrophotometry (AES). Each sample was analyzed in triplicate, concentrations being obtained by interpolation using a calibration curve of absorbance against concentrations of standards. The wavelengths at which the metals were determined and the instrumental conditions used are provided in table 9. In all cases, air- acetylene flame was used.

Table 9. **AAS instrumental conditions and specifications.**

Operating parameter	Cu	Zn	Mn	Fe	K
Wavelength (nm)	324.8	213.9	279.5	248.4	766.5
Slit width (nm)	0.2	0.2	0.2	0.3	0.2
Lamp current (mA)	5	10	10	15	Emission mode
Sensitivity (μg/g)	0.04	0.01	0.04	0.06	0.01
Detection limit (μg/g)	0.005	0.003	0.005	0.05	0.005

3.7.1. **Recovery studies.**

A ground and dried pulp sample was weighed in four portions, 0.5g each and transferred to conical flasks. Three of these were spiked with varying quantities of Cu^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+} and K^{+} ion standard solution. The spiking was done in such away that the standard added corresponded to about the highest, the mean and the lowest concentration of the analyte reported in the samples. This was done in triplicate. They were then digested and analyzed for Cu^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+} and K^{+} ions as described in 3.7 above.

♦ CHAPTER FOUR

4.0. Results and discussion.

4.1. Caffeine content in coffee pulp.

4.1.1. Calibration data.

Plots of peak areas versus concentrations gave straight lines in the concentration ranges 25-200μg/ml and 10-100μg/ml for GC and HPLC respectively (Figure D1 and D2, appendix D). The r-square values were calculated as 0.99728 and 0.98375 for GC and HPLC respectively and the regression equations are;

GC: Peak area = 2353.31 + 929.514 (concentration, μg/ml).

HPLC: Peak area = 11230.8 + 249706 (concentration, μg/ml).

Retention times for caffeine peaks using the two methods were fairly reproducible. For both the GC and HPLC methods, retention times were reproducible up to the first two significant figures. They fell within 1.765±0.035 min. and 9.24±0.05 min. for GC and HPLC respectively.

4.1.2. Minimum Detectable Concentration (MDC).

The minimum detectable concentration (MDC) for caffeine standard by the GC and HPLC method was obtained as 4μg/ml and 0.05μg/ml respectively. The data on table 10 and 11 show how the minimum detectable concentrations were determined. The minimum detectable concentration of 4μg/ml of caffeine obtained using the GC method

is rather high and implies that the method cannot be used to determine caffeine at trace levels. However, at high concentration as in the case of coffee pulp, coffee beans and tea leaves (Clifford, 1991 and Liang *et al*, 1990), the method is acceptable based on the good agreement between the results obtained in this study using GC and HPLC methods.

Table 10. Retention times and peak areas for various concentrations of a caffeine standard (GC).

Caffeineconc(μg/ml).	Retention time(min).	Peak area.
10	1.752	6309
5	1.798	3711
4	1.722	2715
3	-	ND
2	-	ND

Key - = Undetectable.

Table 11. Retention times and peak areas for various concentrations of a caffeine standard (HPLC).

Caffeine conc. (μg/ml)	Retention time(min)	Peak area.
1	9.245	8306
0.5	9.24	4811
0.1	9.235	1401
0.05	9.24	1012
0.025	-	ND
0.01	-	ND

HPLC is a more elaborate method and is reputed for its use in separation and accurate quantitative determination of nonvolatile compounds (Skoog and West, 1982). This is reflected by the higher values for HPLC results compared to the GC results (Table 12). It is for this reason that HPLC has been a traditional method for caffeine analysis. However,

the higher cost of HPLC instrumentation and running costs often makes it not an instrument of choice.

4.1.3. **Caffeine content in fresh coffee pulp.**

The mean caffeine content in the coffee pulp samples varied from 0.22- 0.70 % w/w (dry mass basis) (Table 12). The levels in the marginal coffee zone (UM-3) pulp samples ranged from 0.37-0.70% w/w with a mean of 0.57 % w/w. The levels for UM-1 and UM-2 ranged from 0.29-0.68 and 0.22-0.60 with mean values of 0.49 and 0.39% w/w respectively. Generally, UM-3 pulp samples had the highest caffeine contents followed by UM-1 then UM-2.

Table 12. Mean caffeine content in fresh Kenyan Coffee Pulp (%w/w on dry mass basis) using GC and HPLC.

Agro-Ecological zone.	Factory.	Caffeine Concentration (% w/w)		Mean zonal levels (%W/W)
		GC	HPLC.	
(UM-1).	Kamuchege	0.39±0.034	0.40±0.046	0.49±0.16
	Kirura	0.29±0.017		
	Kanake	0.64±0.041	0.62±0.063	
	Gathiruini	0.68±0.013		
	Githongo	0.46±0.022		
(UM-2).	Mikari	0.41±0.007		0.39±0.13
	Kibonge	0.40±0.008		
	Kagwanja	0.22±0.025		
	Nyagah	0.44±0.031	0.51±0.008	
	Riakahara	0.60±0.032		
(UM-3).	Azania	0.64±0.046	0.65±0.055	0.58±0.14
	Tasia	0.45±0.065		
	Yadini	0.70±0.028	0.76±0.011	
	Ruiru mills	0.69±0.050	0.78±0.076	
	Rukera	0.39±0.005		

These values are within the HPLC data (0.4-1.42 % w/w) for Venezuelan coffee pulp (Clifford and Ramirez-Martinez, 1991), but lower than the value (1.3%w/w) for coffee pulp from Costa Rica (Bressani and Braham,1980).

There were variations in caffeine content of pulp within a zone as well as between zones. These variations can be attributed to differences in the species and cultivars of the coffee trees from which the pulp was obtained, climatic conditions, and soil characteristics. UM-3 had the highest mean zonal caffeine content (0.58% w/w). The plantations in this zone are mainly owned by companies who have access to the capital to purchase the necessary farm inputs such as fertilizers and pesticides. The higher levels of caffeine in the pulp samples from this zone could be due to greater use of nitrogenous fertilizers, since plants in nitrogen rich soils have been shown to be richer in alkaloids than those in nitrogen poor soils (Gartland *et al.*, 1980).

UM-1 and UM-2, both dominated by the cooperative sector had lower mean zonal caffeine contents (0.49 and 0.39 % w/w respectively) than UM-3 (0.58%). This could be due to the fact that some farmers can neglect their coffee especially with regard to fertilizer application. The higher values for UM-1 coffee pulp compared to UM-2 could reflect a better management of coffee farms in this zone. Indeed most pulp samples were obtained from Komothai cooperative societies, which are reputed for their good management. From these results, it appears that well-managed coffee trees will have higher levels of caffeine in the pulp than poorly managed trees.

4.1.4. **Caffeine content in ensiled coffee pulp.**

A comparison between the caffeine content in fresh and ensiled coffee pulp samples is presented in Figure C2, appendix C. The percentage loss of caffeine after ensiling ranged from 30.00 to 45.59% (Table 13). Studies by Bressani and Braham (1970) have also shown that ensiling lowers caffeine content in the pulp.

There is a positive correlation between the values in the fresh and ensiled samples (coefficient of correlation, r = 0.976). A two-tailed t-test on r gave a calculated t-value of 8.963, which is greater than the tabulated value of 4.60 at 99% confidence level. Therefore the null hypothesis that ensiling does not lower the levels of caffeine in the pulp significantly is rejected, implying that it does lower significantly.

Table 13. Mean Caffeine Content in Fresh and Ensiled Coffee Pulp Samples.

Factory	Fresh sample (%w/w).	Ensiled sample (%w/w).	**% Loss of caffeine.**
Gathiruini	0.68±0.013	0.37±0.033	**45.59**
Kibonge	0.40±0.008	0.28±0.028	**30.00**
Kagwanja	0.22±0.025	0.13±0.042	**40.91**
Nyagah	0.44± 0.031	0.27±0.0.024	**38.06**
Tasia	0.45±0.065	0.25±0.032	**44.44**
Yadini	0.70±0.050	0.40±0.048	**42.86**

Nitrogenous compounds are rarely affected by ensiling because lactic acid bacteria, a group of bacteria responsible for the biochemical changes during ensiling, have limited ability to ferment amino acids and other nitrogen containing compounds (Sneath *et al.*, 1986). The observed loss in caffeine upon ensiling is attributed to drainage by the liquids in the drainage liquor. Caffeine is water-soluble and water is a major

constituent in the drainage liquor. Murillo (1974), studied the chemical composition of the drainage liquor of ensiled coffee pulp and reported a concentration of 2.2g/L of caffeine in the liquor. This shows that drainage contributes to the total caffeine loss in ensiled coffee pulp.

The presence of fungi in the silage can also contribute to the overall caffeine loss. The genus *Fusarium* is known to grow on several agro-industrial wastes and produce single cell protein. The growth characteristics of the species *Fusarium oxysporum, Aspergillus oryzae, Paecilomyces elegans* and *Penicillum crustosum* in solid coffee wastes have been studied, and were shown to be able to use chlorogenic acids as a source of carbon and caffeine as a source of nitrogen (Orue and Bahar, 1985). These fungi are not favoured by the conditions of pH and temperature prevailing in these wastes and have to be cultured at their optimum pH and temperature in order to attain the critical fungal population for single cell protein production. There is possibility that the ensiled coffee pulp samples contained some of these fungi which contributed to the biodegradation of caffeine.

The levels of caffeine in both fresh and ensiled coffee pulp samples are within the levels that have been reported to produce antinutritional effects in animals (Bressani and Braham, 1979). It is, however, important to note that ensiling lowers the levels of caffeine by about 40 %. This translates to an increase in the proportion of coffee pulp silage in feed rations compared to the fresh pulp that can be fed to animals without adverse effects.

4.1.5. **Comparison of GC and HPLC results.**

Results for caffeine analysis by GC and HPLC were correlated by the product-moment correlation coefficient (r) to test whether the corresponding values obtained for each sample by the two methods were comparable. The value of r was found to be 0.96. This value lies within the range 0.95< r > 0.99. This shows that there is a good correlation between the values obtained by the two methods. A t- test on the above value of coefficient of correlation (r) using equation A-4 (appendix A), gave a calculated t value of 6.86. Compared to the tabulated value of 4.60 at 99% confidence level, the null hypothesis that there is no significant correlation between the means of caffeine in the pulp samples obtained using the two methods is rejected (since $t_{cal.} > t_{tab.}$). This implies that a significant correlation between the two sets of values exists.

4.2. Tannin content in coffee pulp.

4.2.1. **Calibration Data.**

A plot of absorbance versus concentration of tannic acid gave a straight line (R-square = 0.99157, Figure D4, appendix D) for the concentrations 0.1, 0.2, 0.5 0.8 and 1mg tannic acid/L used. The equation of best line of fit is;

Absorbance = 0.69754 (concentration, mg tannic acid/L) + 0.02706.

Tannin extracts from coffee pulp had an absorption pattern that is similar to that of the tannic acid standard (Figures E10 and E11, appendix E) and therefore the use of tannic acid as a standard for quantitative determination of tannins in coffee pulp is appropriate.

4.2.2. **Tannin content in fresh coffee pulp.**

The total tannin content in the pulp ranged from 1.31-1.64, 1.24-1.45, 1.40-1.50% w/w (on dry mass basis) for UM-1, UM-2 and UM-3 zones respectively (Table 14), with zonal means of 1.55, 1.42 and 1.35%w/w. in that order (Figure C1, appendix C). These values are higher than the values of 0.35-0.68 %w/w for total condensed tannins in coffee pulp reported by Clifford and Ramirez-martinez (1991) using the Butanol-HCl assay. The Butanol-HCl assay is based on the acid catalyzed depolymerization of condensed tannins to give red colored anthocyanidin compounds. This method determines only condensed tannins. In addition, condensed tannins are known to be cleaved in dimers and trimers, which do not absorb as the monomers. The Butanol-HCl assay gives an underestimation of the actual total tannin content. The Folin-Denis assay used in this work determines the total free phenolics, both tannin and non-tannin phenolics. It therefore gives an overestimation of the actual amount of tannins in a sample and the results in Table 14 are expected to be higher than those reported by Clifford and Ramirez-Martinez. They are however, close to that of 1.8%w/w reported by Luiz (1979) using an undisclosed method.

The levels of tannins in coffee pulp from the same agro-ecological zone are close, but there are small variations between samples from different zones. UM-1 has a mean of 1.55% w/w, while UM-2 and UM-3 have means of 1.35 and 1.45 respectively. From the trends above, it seems that environmental conditions such as soil fertility and climate play a major role in the distribution of tannin levels in coffee pulp.

Table 14. Tannin content in fresh Coffee Pulp.

Agro-Ecological zone.	Factory.	%w/w (on dry mass basis)	Mean Zonal levels (%W/W)
UM-1	Kamuchege	1.31±0.057	1.55±0.14
	Kirura	1.56±0.091	
	Kanake	1.48±0.165	
	Gathireini	1.34±0.082	
	Githongo	1.64±0.260	
(UM-2).	Mikari	1.32±0.063	1.35±0.08
	Kibonge	1.45±0.099	
	Kagwanja	1.36±0.017	
	Nyagah	1.32±0.124	
	Riakahara	1.24±0.032	
(UM-3).	Azania	1.46±0.251	1.45±0.04
	Tassia	1.43±0.302	
	Yadini	1.40±0.015	
	Ruiru mills	1.40±0.048	
	Rukera	1.50±0.293	

Environmentally induced stress has been shown to stimulate plants to synthesize more polyphenols in their leaves and fruits. For instance, plants that are exposed to herbivores, pests or pathogens tend to contain more polyphenols, notably tannins (Oates et al., 1977, Gartland *et al.*, 1980). Plants that grow in poor soils or in drier areas are expected to have higher levels of tannins (McKey *et al.*, 1978, Ola *et al.*, 1987 and Gartland, 1980).

The areas covered in this study have relatively rich volcanic soils, with soil fertility, annual rainfall and altitude increasing from UM-3 to UM-1 (Table 8). The tannin levels in coffee pulp reported in this study do not reflect the above trend, but seem to

reflect the extent of exposure of the coffee tree to diseases and insect pests. UM-3 is drier and there is a greater prevalence of insects than in the other zones. On the other hand, there is a greater prevalence of diseases, notably CBD in UM-1 due to the colder climate and higher rainfall (Kenya coffee, 1996). UM-2 is an intermediate zone and has lesser prevalence of diseases and pests. This could explain why UM-1 and UM-3 have relatively higher levels of tannins than UM-2. Tannins are secondary plant metabolites whose role is mainly to offer protection from pests and diseases to the plant (Oates *et al.*, 1977) and the higher levels in UM-1 and UM-3 could reflect a need for protection by the coffee trees.

There was a negative correlation (correlation coefficient, r = -0.5416496) between the levels of caffeine and tannins in the coffee pulp samples from the three agro-ecological zones. A t-test on the above value of r using equation A-4, **appendix A**, gave a calculated t-value of 2.32 which is greater than the tabulated t-value of 2.14 at 95% confidence level, showing that a significant correlation does exist at this level. This could reflect the level of nitrogen in the soil. The negative relationship between the levels of caffeine and tannin levels confirms the proposition that plants growing in nitrogen rich soils tend to synthesize more alkaloids and less polyphenols compared to those that grow in poor soils (Ola *et al.*, 1987 and Gartland, 1980).

4.2.3. **Tannin Content in ensiled coffee pulp.**

The percentage loss of tannins after ensilage ranged from 5.56 to 26.67%w/w (Table 15). A comparison of the levels of tannins in fresh and ensiled pulp samples is presented in Figure C3, appendix C. Polyphenols are toxic to most microorganisms (Ariga *et al.*, 1981) and are not likely to be changed during ensilage by microorganisms.

The loss can be attributed to drainage by the draining liquors. Tannins are water-soluble and can easily be dissolved in and drained along with the liquor. Due to their polymeric nature, they are less soluble in water compared to caffeine and this could explain the lower mean percentage loss for tannins (21.35%) compared to that of caffeine (40.40%).

Although ensiling lowers the levels of total tannins by about 20 %, the levels in fresh and ensiled samples reported in this work are still within the range of 1-2 %w/w that has been shown to exert significant antinutritional effects in most animals (George *et al.*, 1989). However, the results on ensiled samples show an improvement in the proportion of coffee pulp silage in the feeds that can be fed to and accepted by animals.

Table 15. Mean Tannin content in fresh and ensiled Coffee Pulp Samples (%w/w on dry mass basis)

Factory	Fresh sample.	Ensiled sample.	% loss.
Gathiruini	1.34±0.082	1.28±0.114	17.65
Kibonge	1.45±0.099	1.33±0.066	26.67
Kagwanja	1.36±0.017	1.32±0.122	5.56
Nyagah	1.32±0.012	1.24±0.071	21.63
Tassia	1.43±0.302	1.36±0.185	16.28
Yadini	1.40±0.015	1.23±0.026	42.50

Chlorogenic acids in coffee pulp and coffee effluents have been shown to be consumed by some fungi from genus *Fusarium*. This could be the case for other polyphenolic compounds as well. Since chlorogenic are measured as part of the total tannins by the method used in this work, the presence of the said fungi could have contributed to the loss of the tannins after ensiling.

4.3. **Pesticide Residues in Coffee Pulp.**

4.3.1. **Solvent systems and recovery studies.**

In the search for a convenient solvent for multiresidue extraction, different solvent systems were used to extract the various pesticides from spiked samples. By subtracting the concentration values of the pesticides in the unspiked samples from that in the spiked samples, the amounts of pesticides recovered were obtained. The percentage recovery for the different solvent systems for each pesticide was calculated. The results are presented in Table16 below.

Table 16. Mean percentage recoveries for different pesticides using different solvent systems.

Solvent system	Mean recoveries (%)			
	Chlorothalonil	Fenitrothion	Chlorpyrifos	Triadimefon
Toluene	90.08±1.35	92.48±0.22	92.51±0.84	89.33±0.96
Toluene + Hexane (3:1)	96.30±0.46	97.01±0.92	93.73±0.74	95.44±0.53
Toluene + Hexane (1:1)	76.45±0.42	60.41±1.20	56.90±0.96	70.50±0.88
Diethyl ether	81.22±1.10	91.90±0.65	86.41±1.18	90.69±1.05
Diethyl ether-hexane(3:1)	94.60±0.53	93.47±0o49	91.22±0.93	94.33±1.82
Diethyl ether-hexane(1:1)	70.40±0.77	65.92±0.87	60.63±1.18	61.87±0.37

From the results on the Table 16 above, the toluene-hexane (3:1) solvent extracted the highest amounts of the pesticides. However, toluene (Bpt. 111.0 °C) is less volatile than diethyl ether (Bpt. 34.6 °C). A difficulty was experienced in evaporating the solvent prior to the clean up stage. For this reason, the volatile diethyl ether-hexane (3:1) mixture was opted for. All the results reported in this work were obtained by extraction with this solvent system.

4.3.2. **Calibration data and Minimum detectable concentrations (MDC).**

A good correlation between the concentration of the pesticide standards and their corresponding peak areas was observed (Figures D5, D6, D7 and D8, appendix D) The r-square values were 0.99131, 0.99449, 0.99545 and 0.99004 for chlorothalonil, fenitrothion, chlorpyrifos and triadimefon respectively. The equations of the lines of best fit were;

Chlorothalonil: Peak area = 831248 (concentration, µg/mL) + 6410.77

Fenitrothion: Peak area = 189176 (concentration, µg/mL) + 31395.0

Chlorpyrifos: Peak area = 273642 (concentration, µg/mL) + 4118.38

Triadimefon: Peak area = 11792 (concentration, µg/mL) + 31865.5

The minimum detectable concentrations varied significantly from one pesticide to another. They were determined as 0.2, 100, 25, and 4ng/ml for chlorthalonil, fenitrothion, chlorpyrifos and triadimefon respectively as shown in Table 17 below. Chlorothalonil consists of a benzene ring with all the hydrogen atoms substituted by electron capturing groups (-Cl and -CN) and thus its exceptionally low limit of determination using the electron capture detector is expected.

Table 17. Retention times and peak areas of some pesticide standards concentrations.

Chlorothalonil RT.= 2.37±0.01min		Fenitrothion RT.= 3.57±0.01min.		Chlorpyrifos RT.= 4.10±0.01min.		Triadimefon RT.= 4.43±0.02min.	
Conc. (ng/ml)	P.Area	Conc. (ng/ml)	P.Area	Conc. (ng/ml)	P. Area	Conc. (ng/ml)	P. Area
1	259680	400	70223	100	122040	10	36879
0.4	92485	200	34014	50	55049	8	29547
0.2	52006	100	17429	25	25510	4	16254
0.1	-	50	-	12.5	-	2	-
0.05	-	25	-	10	-	1	-

Key

RT = Retention time.

P. Area = Peak area

4.3.3. **Levels of Pesticide Residues in Fresh Coffee Pulp.**

The levels of chlorothalonil, fenitrothion, chlorpyrifos and triadimefon in fresh and ensiled coffee pulp were determined and the results are presented in Table 18. Chlorothalonil was detected in 14 out of the 15 samples, while the other pesticides combined were detected only in 8 out of the 15 samples. It was also noted that chlorothalonil was detected at higher concentrations relative to the other pesticides.

Chlorothalonil is a non-sytemic fungicide and like other organochlorine compounds, it tends to be persistent in the environment. A half-life of up to six months in the soil has been reported (U.S Department of Agriculture, 1994a), but it is expected to be more persistent in the aerial parts of plants. This can partly account for the higher levels of chlorothalonil residues obtained. Organochlorine pesticides are generally less lethal to insects and migroorganisms compared to the organophosphates and the carbamates, and have to be applied at higher doses. In addition, chlorothalonil is applied more frequently, like other contact fungicides for both curative and preventive purposes. Tradimefon is a systemic fungicide, but is recommended to be applied only for curative purposes (Kenya Coffee, 1997). This also contributes to higher residue levels for chlorothanil compared to triadimefon.

Chlorothalonil is only next to the copper-based fungicides in terms of affordability, and for this reason it is very popular with farmers. All these factors

contribute to an increased use of chlorothalonil in the coffee sector and leading to the high levels in the residues reported in this work.

Table 18. Pesticide Residue levels in Fresh and Ensiled Coffee Pulp.

	Concentration (μg/g on dry mass basis).			
	Chlorothalonil	Fenitrothion	Chlorpyrifos	Triadimefon
UM-1				
Kamuchege	36.40±2.39	ND	ND	ND
Kirura	35.72±1.28	ND	ND	ND
Kanake	68.83±0.94	ND	ND	1.41±0.07
Gathiruini	0.02±0.00	ND	ND	ND
Gathiruini**	ND	ND	ND	ND
Githongo	49.26±1.63	ND	ND	ND
UM-2				
Mikari	ND	ND	ND	ND
Kibonge	659.52±6.84	4.68±0.65	ND	3.57±0.31
Kibonge**	1.64±0.04	ND	ND	1.02±0.01
Nyagah	50.46±4.49	ND	ND	ND
Nyagah**	ND	ND	ND	ND
Kagwanja	32.32±2.76	ND	ND	2.66±0.12
Kagwanja**	ND	ND	ND	0.70±0.01
Riakahara	ND	ND	ND	ND
UM-3				
Azania	113.42±5.50	ND	ND	ND
Tassia	311.13±8.02	10.93±0.91	ND	17.33±1.30
Tassia**	70.76±4.66	0.09±0.00	ND	ND
Yadini	1245.02±10.58	ND	14.22±1.09	ND
Yadini**	150.26±6.21	ND	ND	ND
Ruiru mills	21.19±1.47	ND	ND	ND
Rukera	517.66±9.92	0.65±0.01	ND	ND

Key

** = Ensiled sample (ensiling period = 4 months).
NB = Not detected

Fenitrothion and chlorpyrifos, both organophosphorous insecticides, were detected in very few samples and at low concentrations. This can be partly attributed to

their low persistence and partly to the fact that they are applied less frequently at lower concentrations. All insecticides are applied only after cases of insect infestation have been reported in the field (Kenya Coffee, 1997). In some cases insecticides may not be applied at all. The fact that pesticide residues for a given pesticide was not detected in a sample could mean that the pesticide was not applied at all to the coffee trees or the time span between application and picking of cherries was long enough for residues to degrade to undetectable levels. It could also be due to the fact that the instrument was not sensitive enough to detect very low concentrations.

4.3.4. **Pesticide residues in Ensiled Coffee Pulp.**

Upon ensiling, most of the pesticides were reduced to undetectable levels. This loss could have been through the drainage by the silage liquor or microbial enhanced degradation. The latter is expected to predominate since the pesticides have very low solubility in water. The percentage losses ranged from 77.26-100%, 99.18-100%, and 71.43-100% for chlorothalonil, fenitrothion and triadimefon respectively.

Only one sample had chlorpyrifos and it was reduced to an undetectable level after ensiling. The apparently low percentage loss for chlorothalonil could have been due to their high levels in the fresh and partly because of the chemical stability of the compound. The high percentage loses for the organophosphates are expected due to their high susceptibility to hydrolysis.

4.4. Heavy metals and potassium content.

The metals copper, zinc, manganese, iron and potassium are important constituents of the coffee plant nutrition. Potassium is required at macro levels while the others are required at trace levels. They are also required in animal nutrition and their availability in animal feeds can present deficiency or toxicity problems depending on the specific needs of each animal. Sources of trace and ultra-trace metals in the coffee plant are twofold; natural sources (from the soil) and man influenced sources (such as the use of inorganic-based fungicides and fertilizers). Soil is the most important source and their absorption from the soil by the plants depends on soil pH, organic matter content among other factors (Aduanyi, 1973).

In this study, the concentrations of the metals copper, zinc, manganese and iron were determined using atomic absorption specrophotometry (AAS), while potassium was determined using atomic emission spectrophotometry (AES). The results are presented in Table 19. The Calibration curves for the respective metal ion standards used are provided in Figures D9 to D13, appendix D. In order to test the efficiency of the digestion process and the precision of the method, recovery studies were carried out on spiked samples. The amounts of each metal recovered were obtained by subtracting the values obtained in the unspiked samples from those in the spiked samples. The percentage recoveries averaged 99,0%, 98.7%, 102.1%, 101.6% and 98.1% for Cu, Zn, Mn, Fe and K respectively. Within experimental limits, these values indicate complete recovery and clearly show the superiority of AAS technique in the determination of metal ions.

All the metals were detected at appreciable levels. This is expected since all the metals are essential nutrients in the coffee plant nutrition and indeed all plants. In

addition, the areas where samples were collected from have red volcanic soils that are rich in mineral matter.

The distribution of copper is most erratic among the metals. Copper levels fall in the range 4.90- 213.30 μg/g, with an overall mean of 44.25 (n = 15) and a standard deviation of 53.89. UM-1 has a zonal mean of 49.58μg/g, UM-2 a mean of 5.35 and UM-3 a mean of 39.24. Even within a zone there are large variations as in the cases of Githongo, Riakahara and Rukera samples. These variations arise from differences in soil characteristics and composition, the duration between the time of picking and the last spray of copper-based fungicides, amount of copper sprays and the prevailing weather conditions between the spraying period and picking of the cherries among other factors.

Table 19. Selected metal ions' content in Kenyan Coffee pulp.

	Concentration (μg/g± sd). (n = 3)				% K db± sd
Factory	Copper	Zinc	Manganese	Iron	Potassium
UM-1					
Kamuchege	43.38±0.28	28.03±2.45	22.31±0.26	90.18±2.73	2.35±0.01
Kirura	14.11±0.36	22.71±0.40	5.88±0.22	87.43±0.78	1.07±0.19
Kanake	47.40±1.06	11.23±0.17	7.75±0.22	46.86±0.05	0.90±0.02
Gathiruini	93.44±1.32	17.09±0.433	13.58±0.80	67.52±1.03	3.32±0.68
Githongo	213.30±2.35	12.61±0.32	35.85±0.96	65.39±0.37	4.21±0.03
UM-2					
Mikari	4.90±0.13	15.91±0.58	119.29±1.15	452.81±1.13	3.75±0.04
Kibonge	5.98±0.15	16.55±0.23	7.30±0.01	152.94±3.35	3.93±0.04
Nyagah	4.75±0.04	10.51±0.47	69.84±2.16	60.09±0.47	3.87±0.06
Kagwanja	5.36±0.16	13.42±0.07	82.19±0.52	154.78±1.15	4.16±0.06
Riakahara	68.80±1.67	22.49±0.85	12.31±0.26	65.21±2.27	2.57±0.03
UM-3					
Azania	23.38±0.26	14.99±0.17	3.49±0.16	44.72±0.83	1.57±0.04
Tasia	42.15±0.89	11.47±0.07	6.26±0.14	180.93±1.81	1.80±0.04
Yadini	53.04±1.32	12.04±0.055	5.81±0.06	112.66±0.51	2.21±0.03
Ruiru mills	38.42±0.77	14.44±0.25	5.23±0.20	12.53±0.07	1.45±0.04
Rukera	5.34±0.11	14.13±0.18	92.29±1.38	107.37±0.36	1.87±0.03

Samples from UM-1 had generally high copper concentrations in the pulp. This zone is colder and wetter; conditions that favour the fungi that cause coffee berry disease (CBD) (Waller, 1987). Fungal diseases are most prevalent in this region and farmers are forced to use copper-based fungicides in the management of these diseases. This partly explains the high copper levels reported in this zone. The exceptionally high level for the Githongo sample could be due to a short time lapse between spraying and picking and possibly the absence of rainfall within the same period. Indeed most of the copper was adsorbed on the surface of the pulp because after washing the pulp samples with 0.1M HCl, about 42.0% of the total copper was lost (Table 20).

UM-2 samples had copper levels in the range of 4.75-5.98µg/g, except for the Riakahara sample, which had a much higher value of 68.80µg/g. CBD is least prevalent in this region and therefore copper sprays are rarely used. Thus the value of 5µg/g could correspond to the level of copper in the pulp that is almost wholly derived from the soil. This is slightly below the range (7-20µg/g) in the leaves required by the coffee tree for optimal growth (Willson, 1985).

The levels for UM-3 samples were also high, and this can be attributed to the fact that most of the farms in this zone belong to big companies who have the capital to buy the necessary farm inputs such as the copper fungicides. The percentage loss of copper after washing the samples ranged from 6.65- 42.00%, implying that most of the copper occurred in the pulp tissues. The regular use of copper-based fungicides combined with the use coffee pulp as mulch could have led to an accumulation of copper in the soil and consequently soil-derived copper in the coffee tress.

Table 20. Mean Copper ion levels in Unwashed and washed Coffee Pulp Samples.

	Concentration (μg/g ± sd).		% loss of copper.
Factory.	Unwashed sample	Washed sample	
Gathireini	93.44±1.33	59.29±2.48	36.55
Githongo	213.30±2.35	123.72±3.05	42.00
Mikari	4.90±0.13	4.05±0.10	17.33
Kagwanja	5.36±0.16	5.02±0.08	6.50
Yadini	53.04±1.32	47.38±0.22	10.67

From the results on Table 19 above and the comparison presented in Figure C4, appendix C, it is evident that washing alone can remove a significant amount of copper in the coffee pulp. It is therefore possible that most of the surface bound copper had been washed away along with the large amounts of water used in the depulping process.

Levels of potassium ranged between 0.90 to 4.21%w/w. These values are consistent with that for coffee pulp from Costa Rica (1.76% w/w) reported by Bressani (1972). These values are expected since potassium is a macroelement essential for the vegetative growth of plants. It is usually supplied to the soil in form of NPK fertilizers of various formulations.

The potassium content in the pulp samples varied with the location. Values for each zone were very close with the zonal means being 2.74, 3.66 and 1.78% w/w (n = 5) for UM-1, UM-2 and UM-3 respectively. The amount of potassium in UM-3 soils is low compared to the other zones and NPK fertilizers have to be applied to maintain the

potassium balance in the soil (Kenya coffee, 1996). Coffee pulp is used extensively as mulch in most estates of UM-3 due to its high potassium content.

There are no extreme variations in the levels of the other heavy metal elements within a zone, especially for zinc, implying that soil characteristics and composition plays an important role in their distribution. UM-1 had a mean zinc level of 18.33μg/g, UM-2 a mean of 15.78 and UM-3 a mean of 13.41μg/g (n = 4). A similar trend is observed for the other metals, except in some cases of iron and manganese. This implies that the three agro-ecological zones do not differ significantly in their metal ion contents since the metal concentrations reported in the pulp are basically derived from the soil.

Copper has low mammalian toxicity. . The copper tolerance level for cattle is 115μg/g of copper in daily diet (Suttle and Price, 1976) and in some cases such as pigs, it is required at much higher concentrations (200-400μg/g) to stimulate growth. Zinc, manganese and iron also have low mammalian toxicities. There are no clearly set guidelines for the allowed limits of these metals in animal rations since the gap between toxicity levels and the levels required for animal growth is wide and for most animals, a level of up to 1000μg/g is still considered safe (MacDonald *et al.*, 1990). The levels of heavy metals (Table 19) are generally safe for most animals. Only one sample exceeded the level for copper considered detrimental to cattle.

4.5. Conclusions and Recommendations.

4.5.1. Conclusions.

At the present time the Kenyan coffee industry is experiencing severe crisis due to falling world coffee prices, consequently this agroindustry needs to add value to its by-products in order to remain economically viable. It is in light of this need that this study was carried out to explore the possibility of using coffee pulp as animal feed. It basically involved the identification and determination of some toxic and antiphysiological elements and compounds in coffee pulp.

From the results obtained in this study it is deduced that the antinutritional substances; caffeine and tannins are present in coffee pulp at levels well above trace amounts. Copper, zinc, manganese, iron, potassium and pesticide residues are also present at appreciable amounts. Based on the distribution of the metal ion levels in the samples, it was evident that copper is a principal contaminant of Kenyan coffee pulp. The other metals are basically derived from the soil, and soil characteristics and composition play an important role in their distribution. Among the pesticides considered, chlorothalonil was a major contaminant.

Owing to the present limited knowledge on the physiological effects on animals of the antinutritional substances determined, no tolerance levels have been set for different animals. However, based on the qualitative information available (Bressani, 1979) and the results of this work, it is admitted that Kenyan coffee pulp is contaminated with toxic and antinutritional substances and any effort to convert coffee pulp into an animal feed must begin by addressing the issue of decontamination.

The levels of caffeine, total tannins and pesticide residues in the pulp reduced significantly after ensiling. Caffeine was reduced by an average of 40.40% (n = 6), tannins by an average of 21.35% (n = 6) and the pesticide residues by between 70-100% (n = 6). It

is therefore deduced that ensiling is an important method of reducing the levels of these substances in the pulp.

Copper, zinc, manganese and iron have low mammalian toxicity (MacDonald *et al.*, 1987) and may not be considered a major hindrance in the use of coffee pulp as animal feed. The role of potassium has not been fully understood, but it is not lethal to animals. Based on the available information, it is concluded that coffee pulp silage can be used as animal feed at supplemental levels with other forages such as nappier grass and mollases. This can only be done after the isolation and characterization of pesticide metabolites in coffee pulp and their toxicity having been fully defined. Supplementing coffee pulp with other forages will tend to dilute the concentrations of the undesirable substances and make the feed more acceptable to the animal.

4.5.2. **Recommendations and areas for further research.**

The intention of this work was principally exploratory. Alot is yet to be done before an acceptable coffee pulp meal product is availed. Therefore the following recommendations and areas of further research are suggested.

1. There is need to isolate and characterize the degradation products of all the pesticide residues present in fresh and ensiled coffee pulp and also determine their toxicity to animals and man. This is because in some cases pesticides may degrade to more toxic compounds.
2. A pilot scale ensiling of coffee pulp needs to be caried out and its effect on the levels of caffeine, polyphenols and pesticide residues investigated. The present work investigated the effect of laboratory scale ensiling. Although the principle is the same, there are some technical variations and this may be reflected in the extent of degradation of the above substances.

3. Once a pilot scale ensiling proves successful and the residual levels of the toxic substances considered safe, field trials on animals should be carried out. This will involve investigations into the voluntary intake of the pulp silage by animals, conversion efficiencies and determination of the levels of pesticide residues in milk and meat products.
4. The levels of pesticide residues in coffee pulp were appreciable. Some of the pesticides used in the Kenyan Coffee industry are systemic in nature, there is possibility that they could also be present in the coffee beans. This can pose a health risk to consumers of coffee. It is necessary to determine the levels of pesticides and their metabolites in both coffee beans and the finished coffee with a view of finding out their implication on human health.
5. During field application, most of the pesticides end up in the soil. In addition, most of the coffee pulp is currently used directly as mulch. As reported in this study, the pulp is fortified with pesticides and a build up of the same in the soil could arise. This could tilt the soil microbial balance and consequently impact negatively on soil fertility. An assessment of the levels of pesticide residues and their metabolites in the soil needs to be carried out. An investigation into the effects of these residues on the various soil parameters should then follow.
6. Since the levels of heavy metals studied in this work were found to be safe, there is need to check if the same holds for other heavy metals especially lead, cadmium and mercury, which are known to be highly toxic at very low concentrations.

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Appendix A.

Statistical Formulae.

1. **Mean.**

The mean levels of each substance for each sample were calculated for triplicate or duplicate determinations to accuracy into the actual value of the levels. The mean zonal value for each substance was also calculated. This was done using equation A-1. Where x_i is the ith term of the determination or the set of data and n is the number of

$$\bar{x} = \frac{\sum_{i=1}^{i=n} \left(x_i - \bar{x}\right)}{n} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots A-1$$

determinations or data.

1. Standard deviation, S.

This was used to measure dispersion of values about the mean. Equation A-2 was

$$S = \sqrt{\frac{\sum_{i=1}^{i=n} \left(x_i - \bar{x}\right)^2}{n-1}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots A-2$$

used to calculate the standard deviation.

2. Correlation coefficient, r.

The pearson (product moment) correlation coefficient, r was used to test linearity within the concentration ranges used for the standard in the preparation of calibration curves. The relationships between the levels of the various substances determined using the r values obtained using equation A-3.

$$r = \frac{\sum_{i=1}^{i=n}\left(x_i-\bar{x}\right)^2\left(y_i-\bar{y}\right)^2}{\sqrt{\sum_{i=1}^{i=n}\left(x_i-\bar{x}\right)^2\left(y_i-\bar{y}\right)^2}} \qquad \text{.......................................} A-3$$

Where x and y represent the two parameters or the two sets of data being correlated and n is the number of the values.

The degree of correlation is treated as fair for $0.90 < r > 0.95$, good for $0.95 < r > 0.90$ and excellent for $r > 0.99$.

3. t-Test on the r-value.

The statistical test used to test whether the correlations obtained between the levels of the various substances determined is t-test. The value of t was calculated using equation A-4

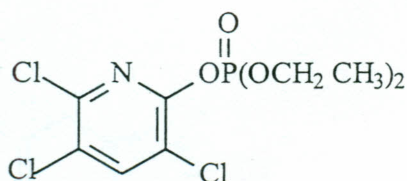
$$t = |r|\sqrt{\frac{(n-2)}{(1-r^2)}} \qquad \text{.......................................} A-4$$

Where r is the coefficient of correlation between the two sets of data.

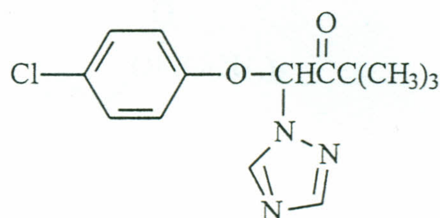
The t-values calculated using equation A-4 were compared with tabulated t-values at various confidence levels using a two-tailed test and (n-2) degrees of freedom. The null hypothesis was that there is no correlation between the levels of the substances determined. If $t_{calculated} > t_{tabulated}$, the null hypothesis is rejected, implying that a correlation does exist. If $t_{calculated} < t_{tabulated}$, the null hypothesis is accepted, implying that a relationship does not exist.

Appendix B.

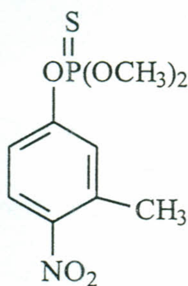
The structures of some of the organic-based pesticides used in the control of coffee pests and diseases.



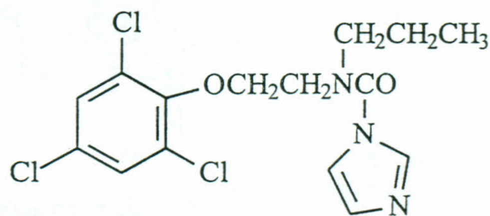
Chlorpyrifos.



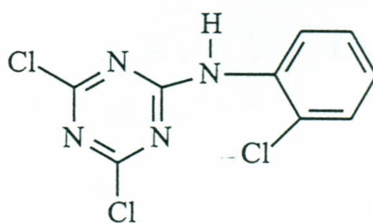
Triadimefon.



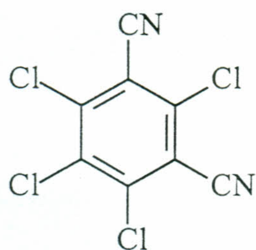
Fenitrothion.



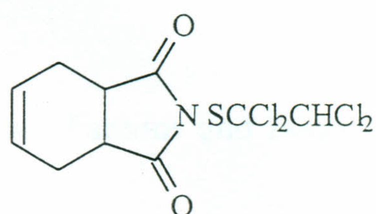
Prochloraz.



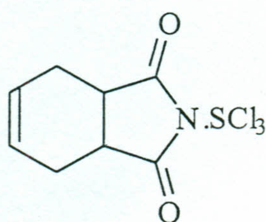
Anilazine.



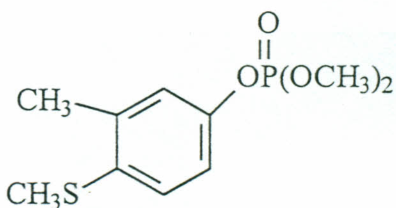
Chlorothalonil.



Captafol.



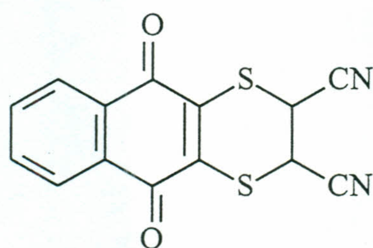
Captan.



Fenthion.

Dimethoate.

Hexaconizole.



Dithianon.

Ethion.

APPENDIX C.
Bar graphs.

Figure C1. Mean zonal levels of Caffeine, Tannins and Potassium in fresh Kenyan coffee pulp.

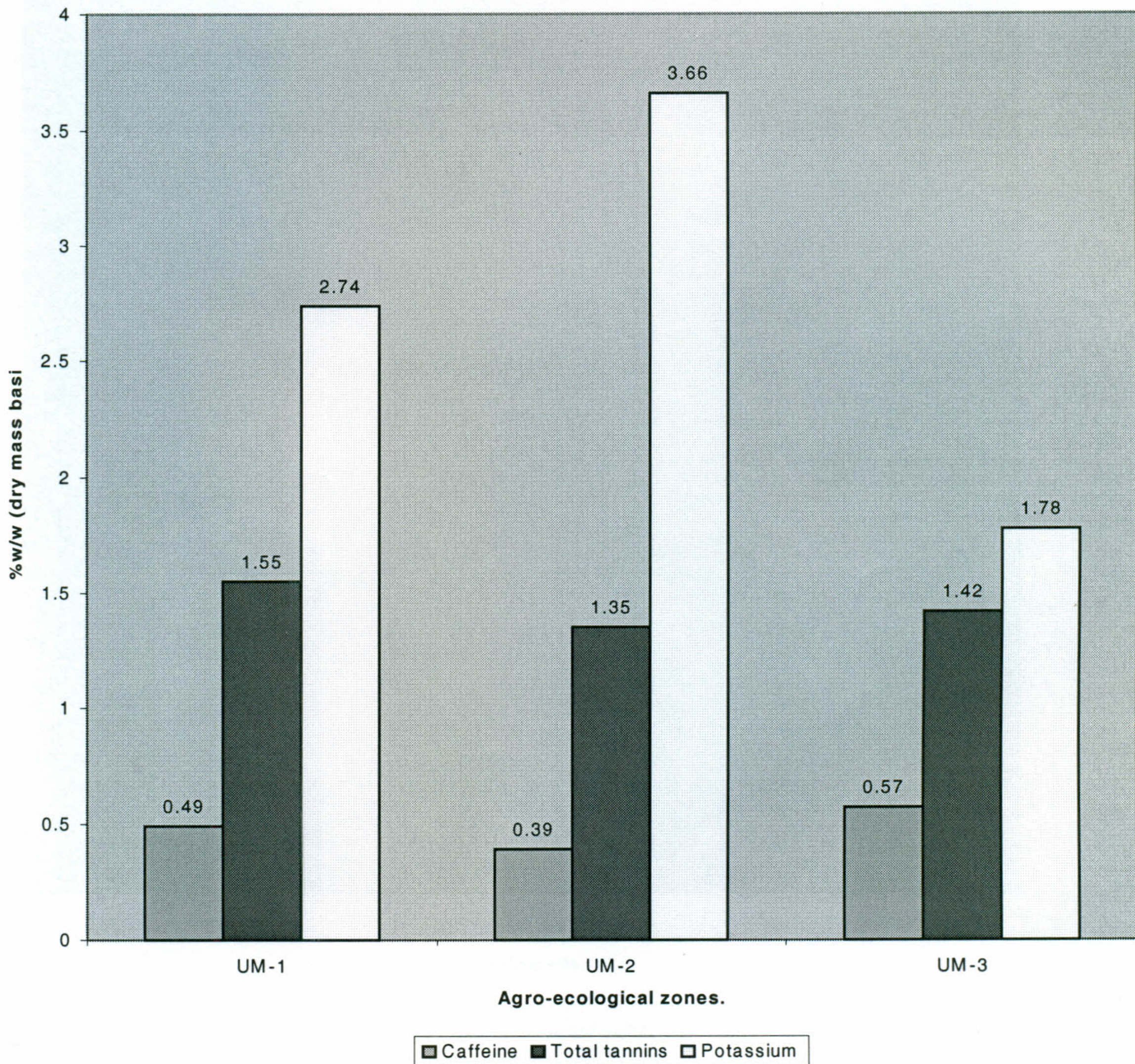
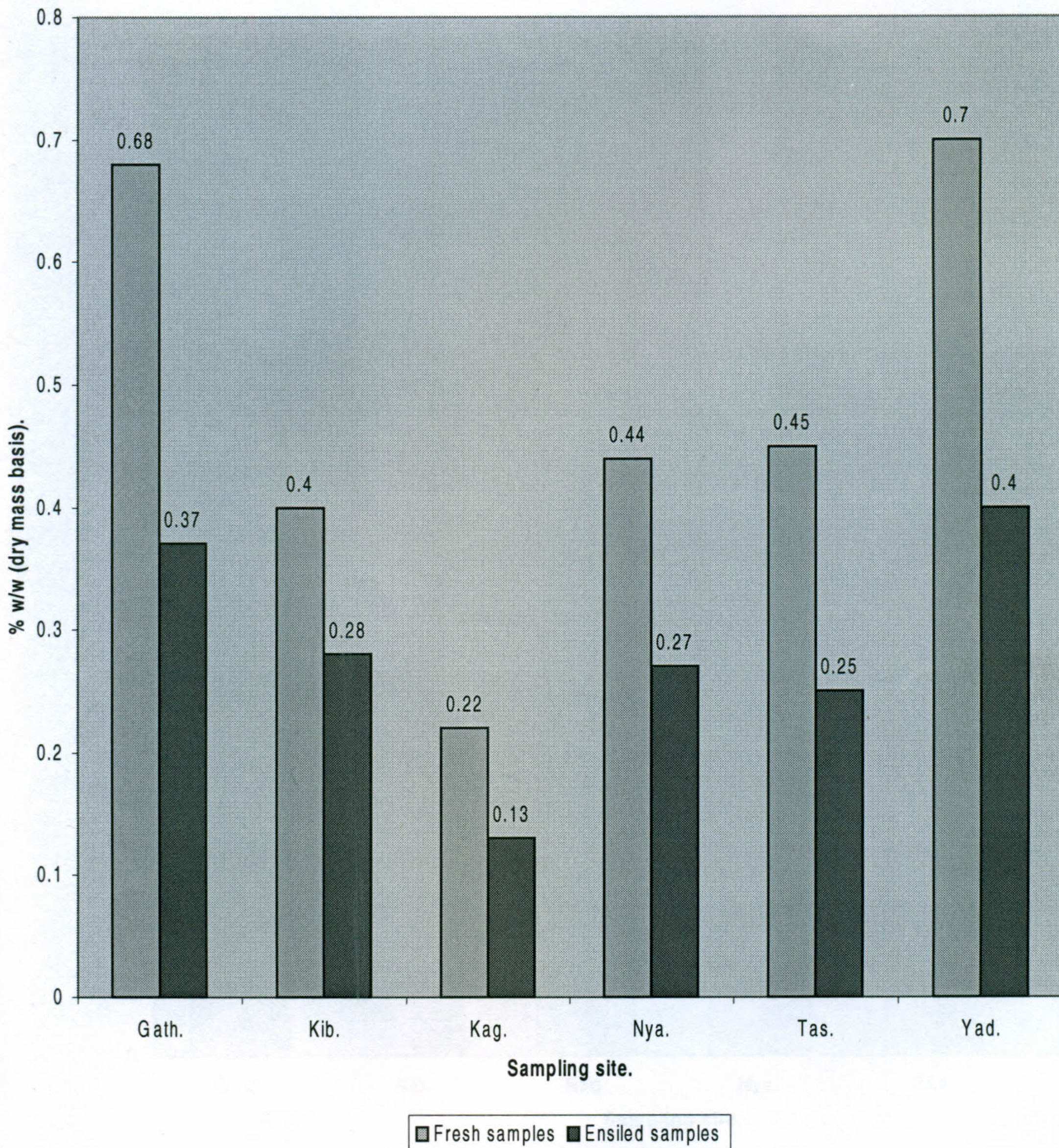


Figure C2. Mean caffeine levels in Fresh and Ensiled coffee pulp samples.



Key

Gath. = Gathuruini, Kib. = Kibonge, Kag. = Kagwanja
Nya. = Nyangah, Tas. = Tassia, Yad. = Yadini

Figure C3. Mean tannin levels in Fresh and Ensiled coffee pulp samples.

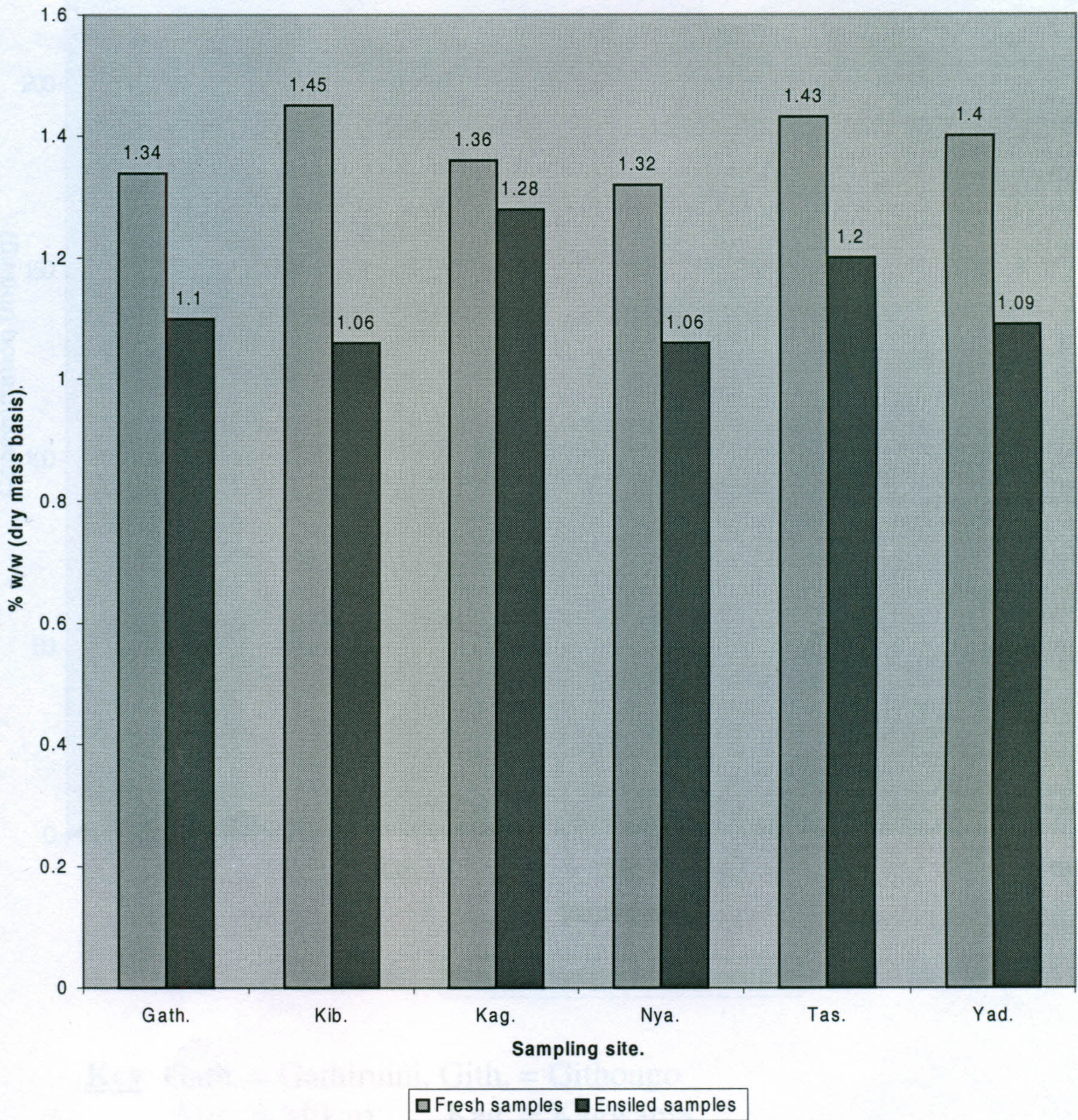
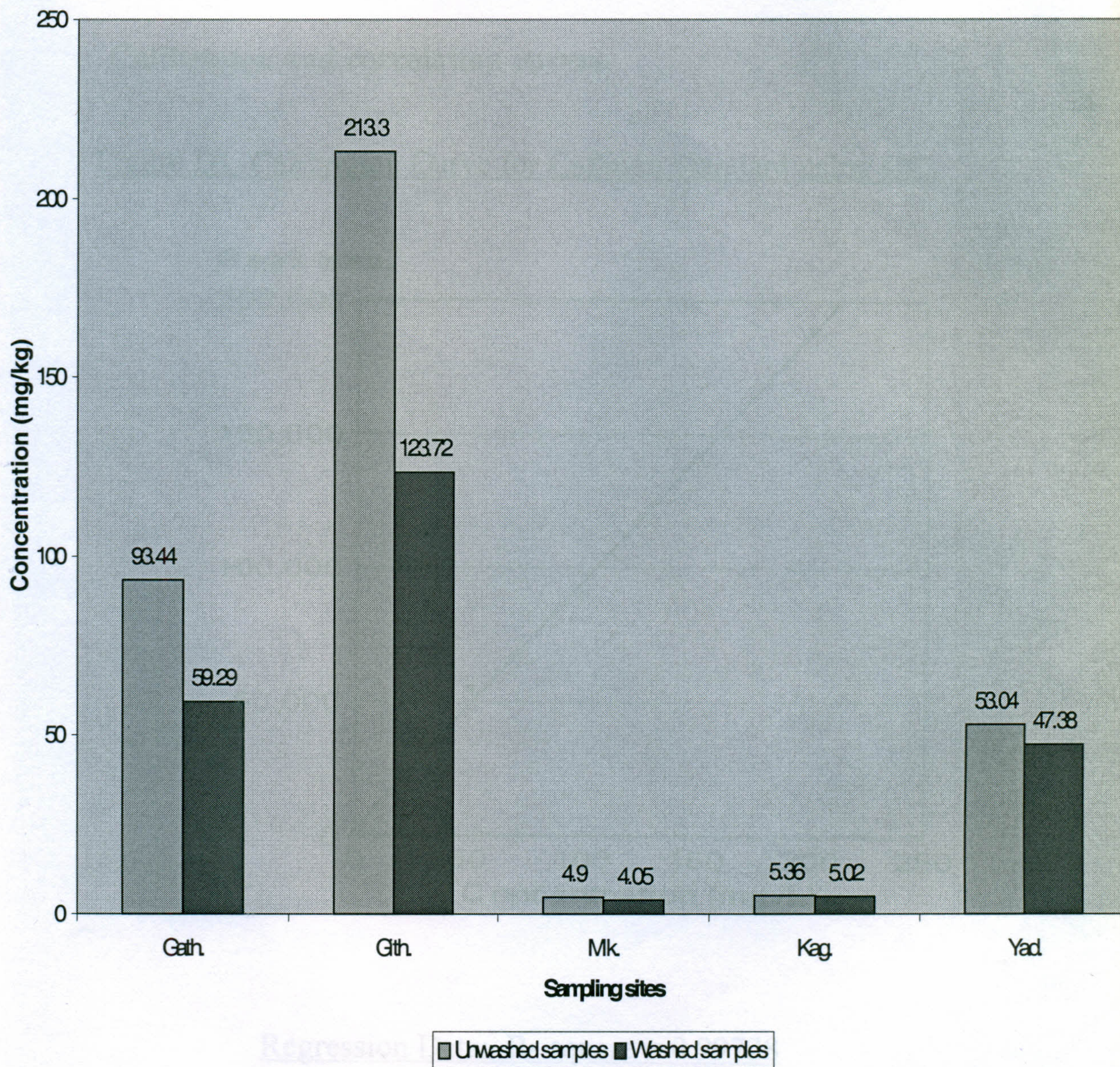


Figure C4. Mean copper levels in Unwashed and Washed coffee pulp samples.

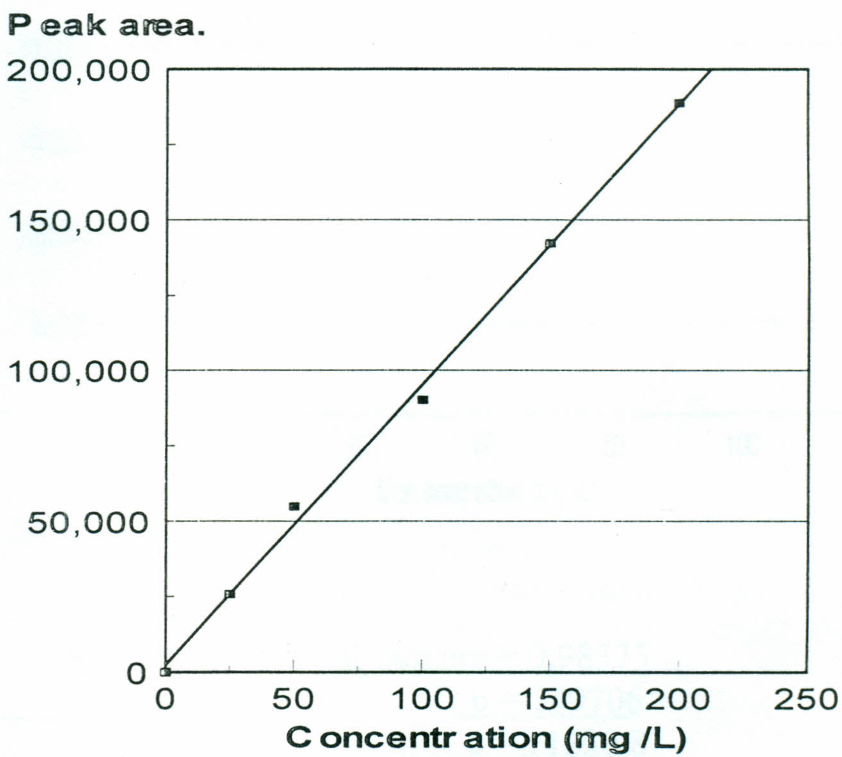


Key: Gath. = Gathiruini, Gith. = Githongo
Mik. = Mikari Kag. = Kagwanja
Yad. =Yadini

APPENDIX D.

Calibration and correlation curves:-

Figure D1. Calibration Curve for Caffeine Standard using GC.



Regression Data: R- square = 0.99728

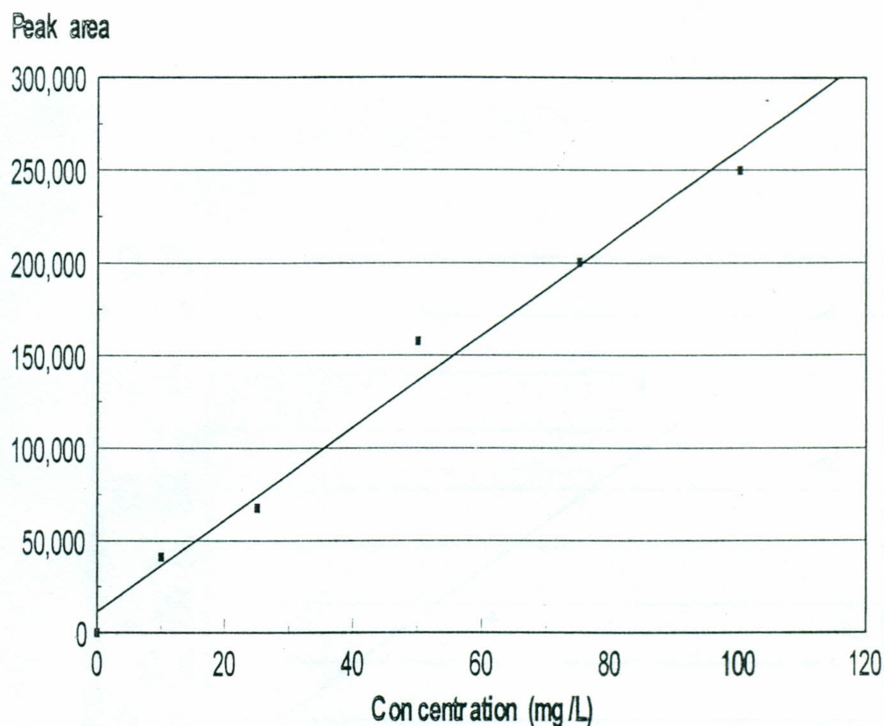
b = 929.514

a = 2353.31

Regression equation.

Peak area = 929.514 (concentration) + 2353.31

Figure D2. Calibration Curve for Caffeine Standard using HPLC.



Regression Data: R- square = 0.98375

b = 249706

a = 11230.8

Regression equation.

Peak area = 249706 (concentration) + 11230.8

Figure D3. Correlation curve for values obtained in the determination of caffeine in Coffee Pulp using HPLC and GC.

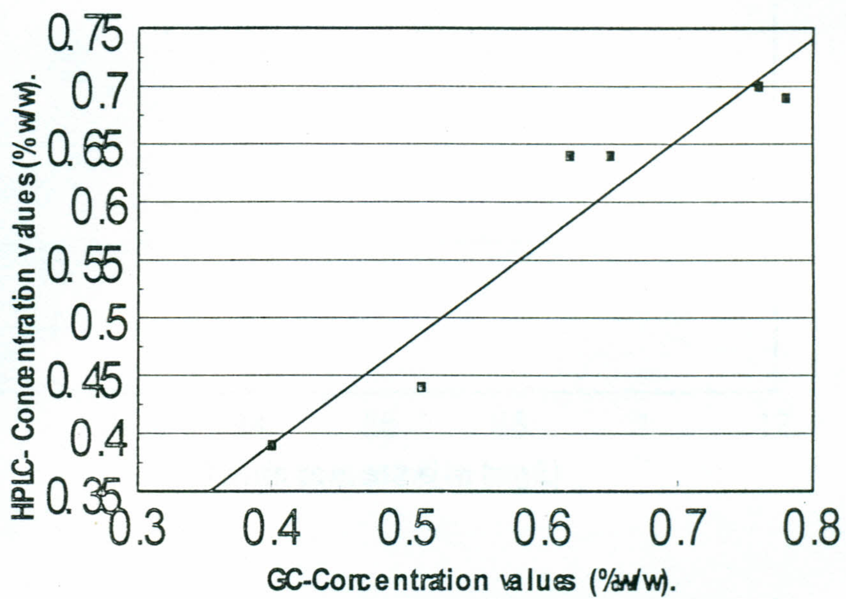
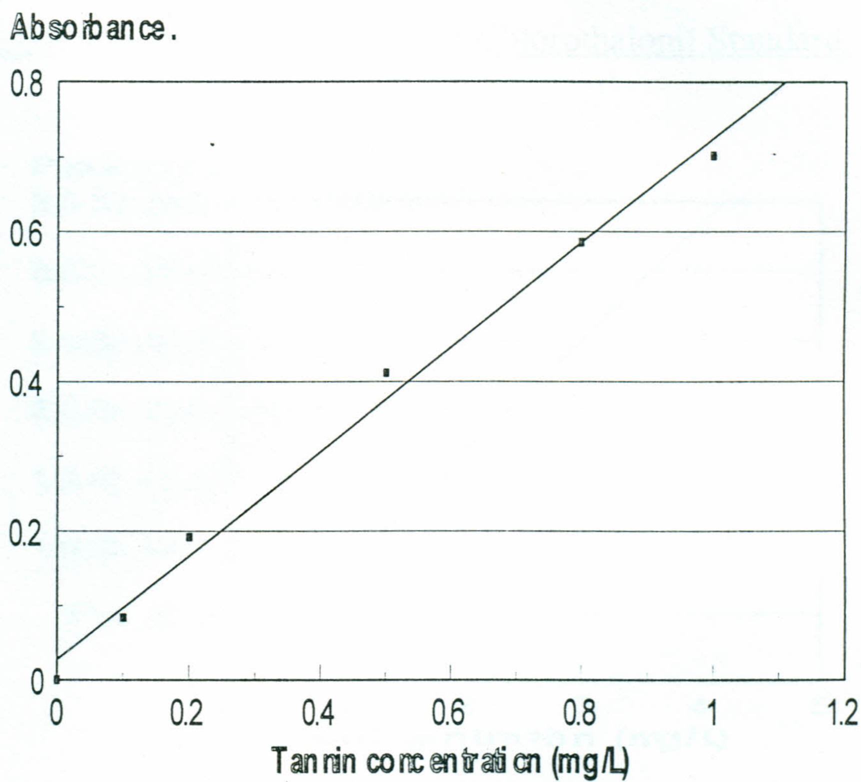


Figure D4. Calibration Curve for Tannic acid Standard.



Regression Data: R-square = 0.99157

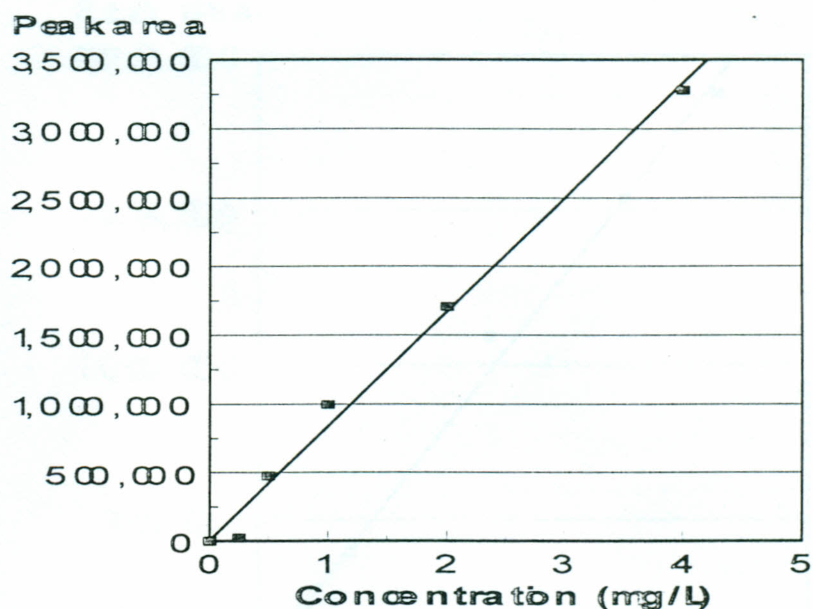
b = 0.69754

a = 0.02706

Regression equation.

Absorbance = 0.69754 (concentration) + 0.0270

Figure D5. Calibration Curve for Chlorothalonil Standard.



Regression Data: R-square = 0.99131

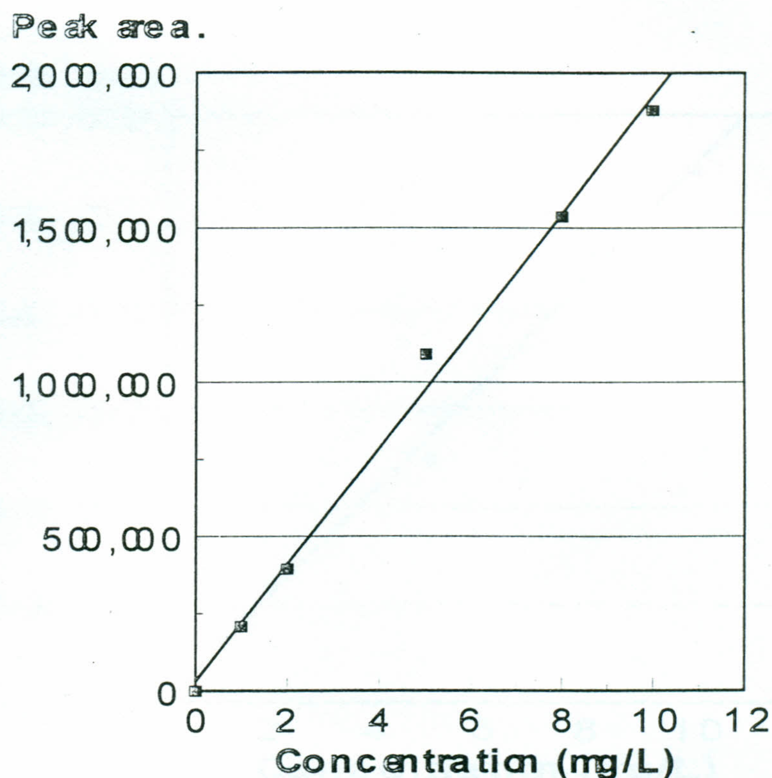
b = 831248

a = 6410.77

Regression equation.

Peak area = 831248 (concentration) + 6410.77

Figure D6. Calibration Curve for Fenitrothion Standard.



Regression Data: R-square = 0.99449

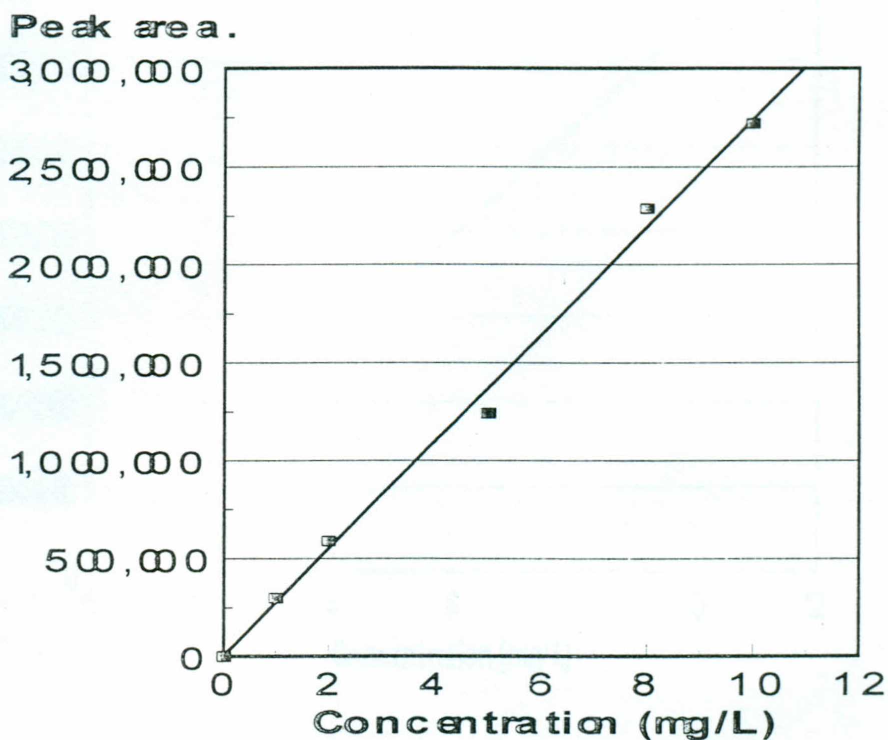
b = 189176

a = 31395.0

Regression equation.

Peak area = 189176 (concentration) + 31395.0

Figure D7. Calibration Curve for Chlorpyrifos Standard.



Regression Data: R-square = 0.99545

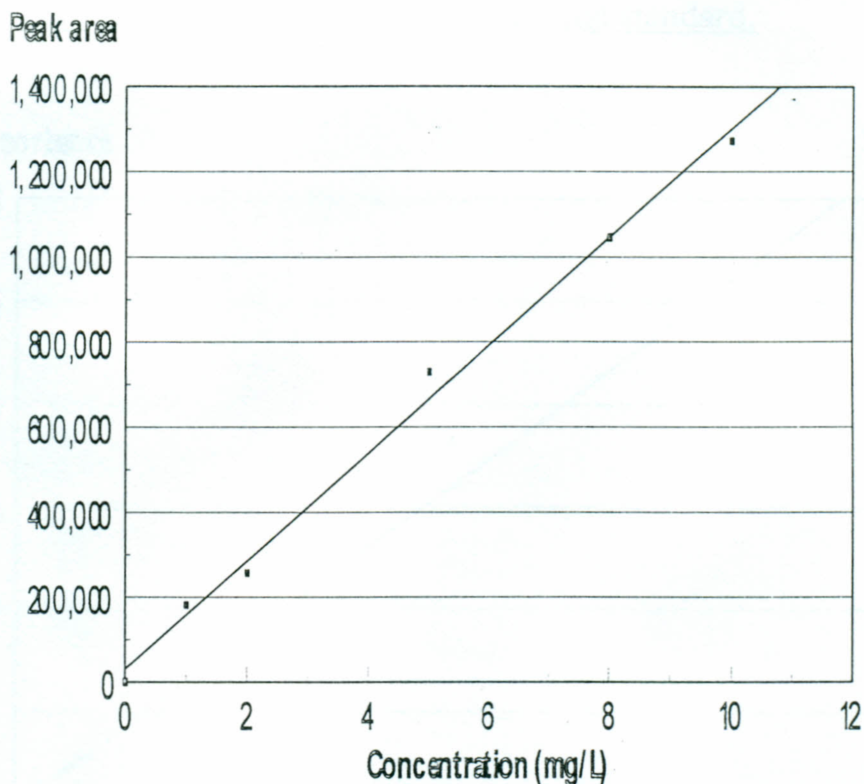
b = 273642

a = 4118.38

Regression equation.

Peak area = 273642 (concentration) + 4118.38

Figure D8. Calibration Curve for Triadimefon Standard.



Regression Data: R-square = 0.99004

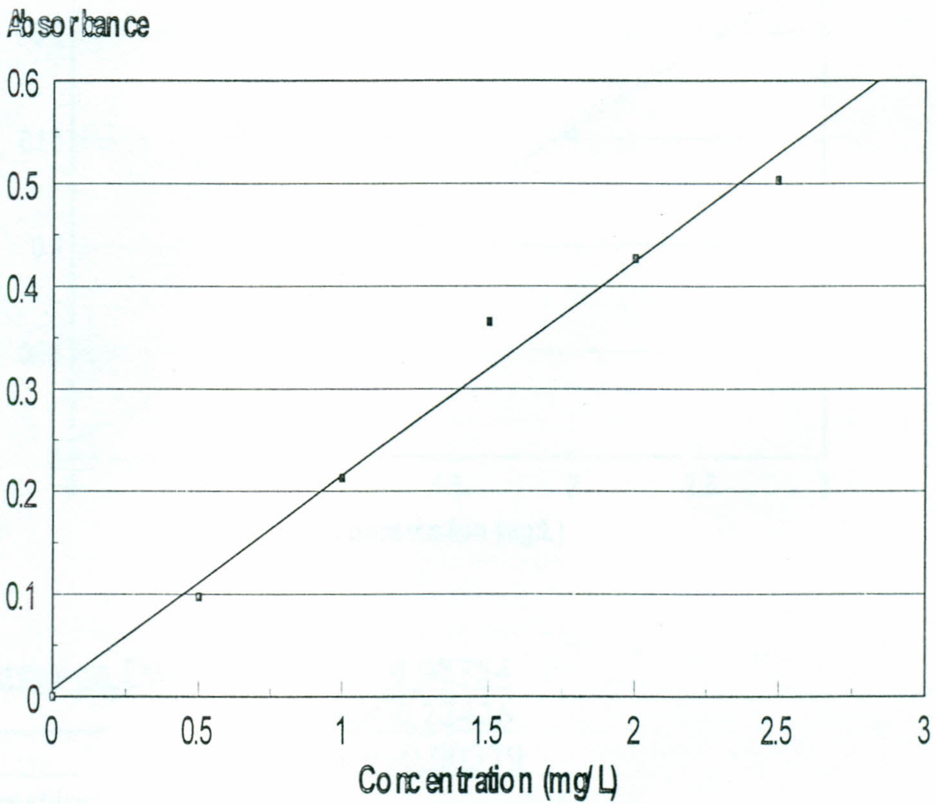
b = 11792

a = 31865.5

Regression equation.

Peak area = 11792 (concentration) + 31865.5

Figure D9 Calibration curve for Cu^{2+} ion standard.



Regression Data: R-square = 0.98478

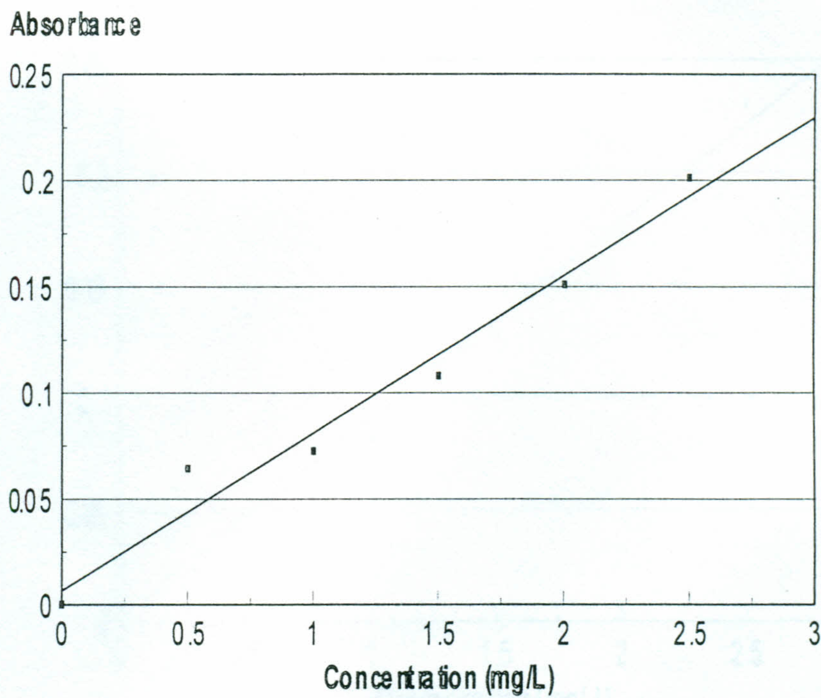
b = 0.20896

a = 0.00640

Regression equation.

Absorbance = 0.20896 (concentration) + 0.00640

Figure D10. Calibration curve for Zn^{2+} ion standard.



Regression Data: R-square = 0.98754

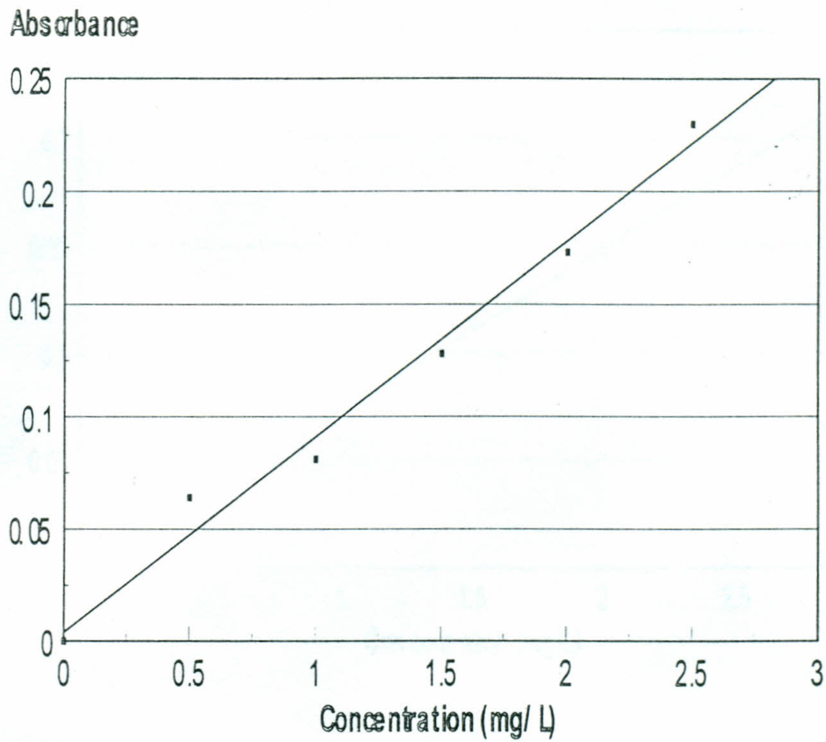
b = 0.22426

a = -0.00379

Regression equation.

Absorbance = 0.22426 (concentration) - 0.00379

Figure D11. Calibration curve for Mn^{2+} ion standard.



Regression Data: R-square = 0.97026

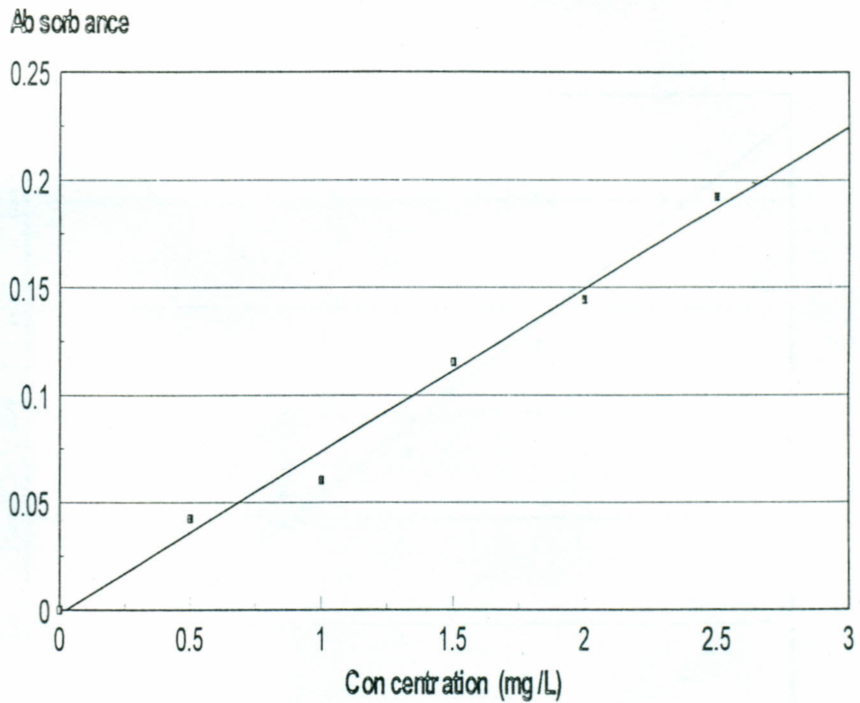
b = 0.08797

a = 0.00646

Regression equation.

Absorbance = 0.08797(concentration) + 0.00646

Figure D12. Calibration curve for Fe^{2+} ion standard.



Regression Data: R-square = 0.98831

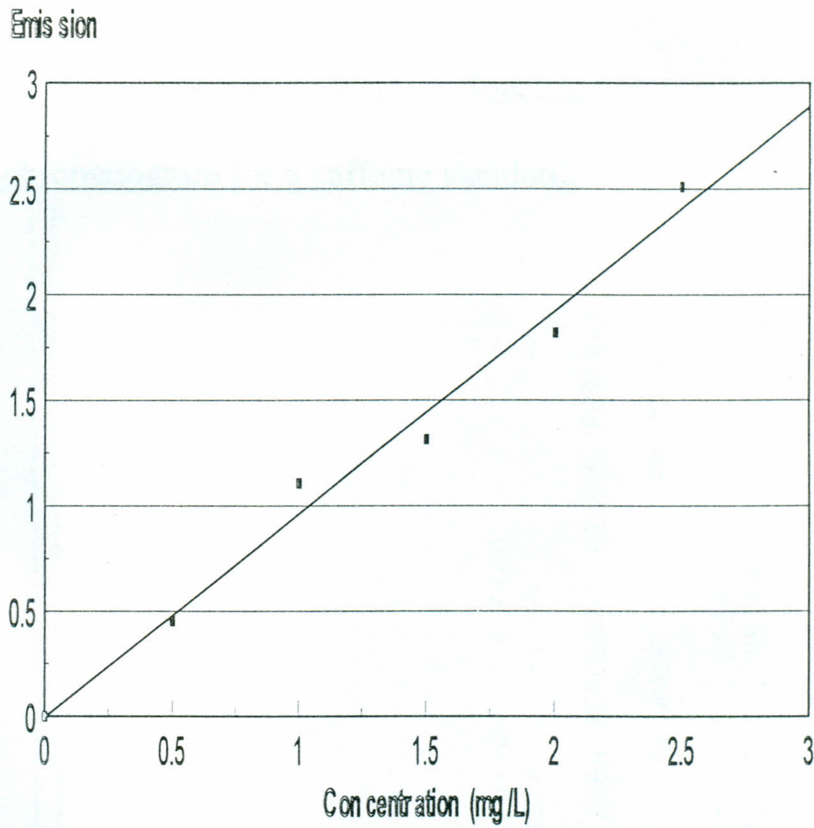
b = 0.07550

a = -0.00189

Regression equation.

Absorbance = 0.07550 (concentration) - 0.00189

Figure D13. Calibration curve for K^+ ion standard.



Regression Data: R-square = 0.98550

b = 0.96422

a = -0.00461

Regression equation.

Emission = 0.98550 (concentration) - 0.00461

APPENDIX E.

Chromatograms and Absorption Spectra.

Figure E1. GC chromatogram for a caffeine standard.

Figure E2. GC chromatogram for caffeine extract of Rukera coffee pulp sample.

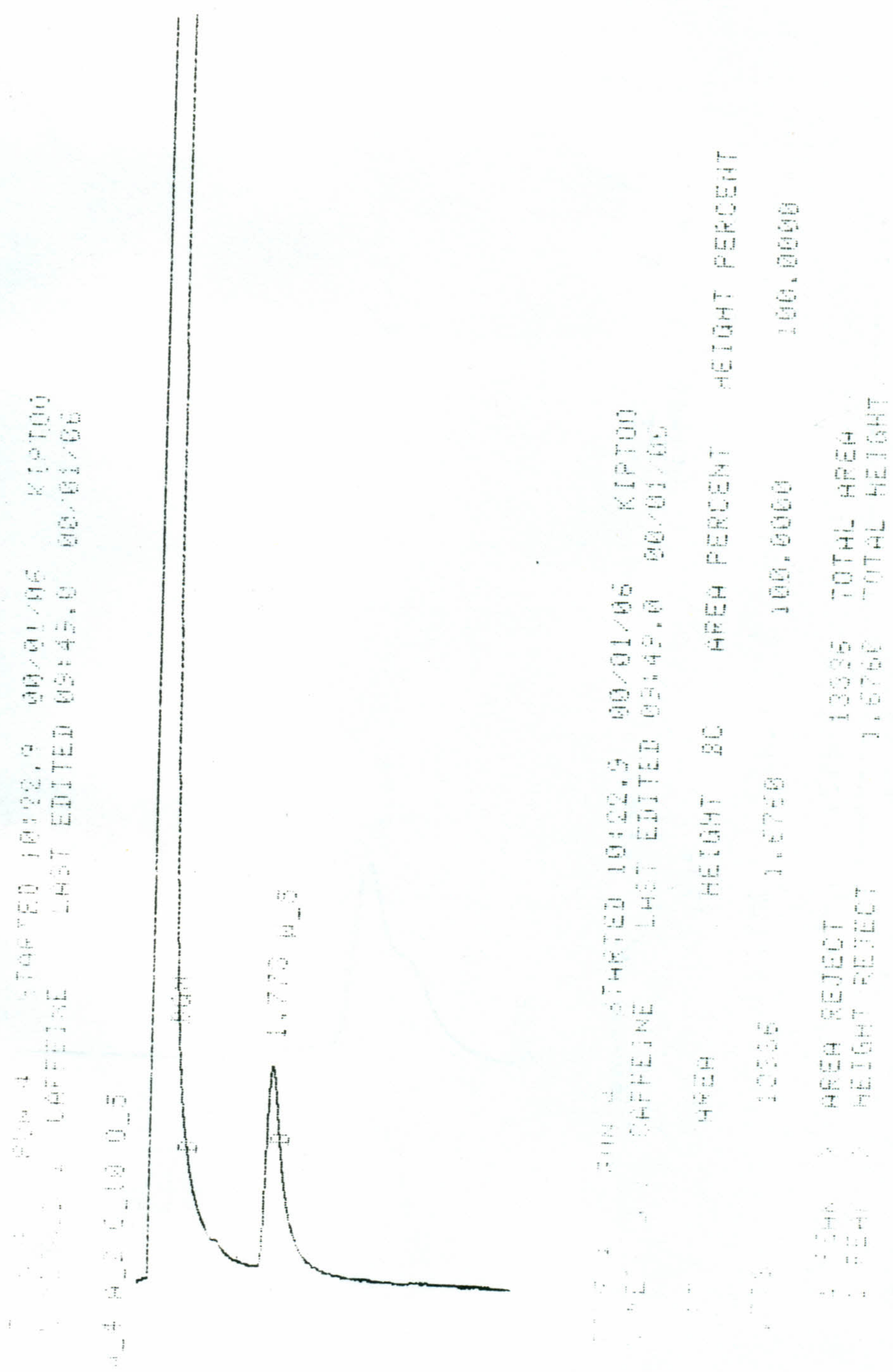


Figure E3. HPLC chromatogram for a caffeine standard.

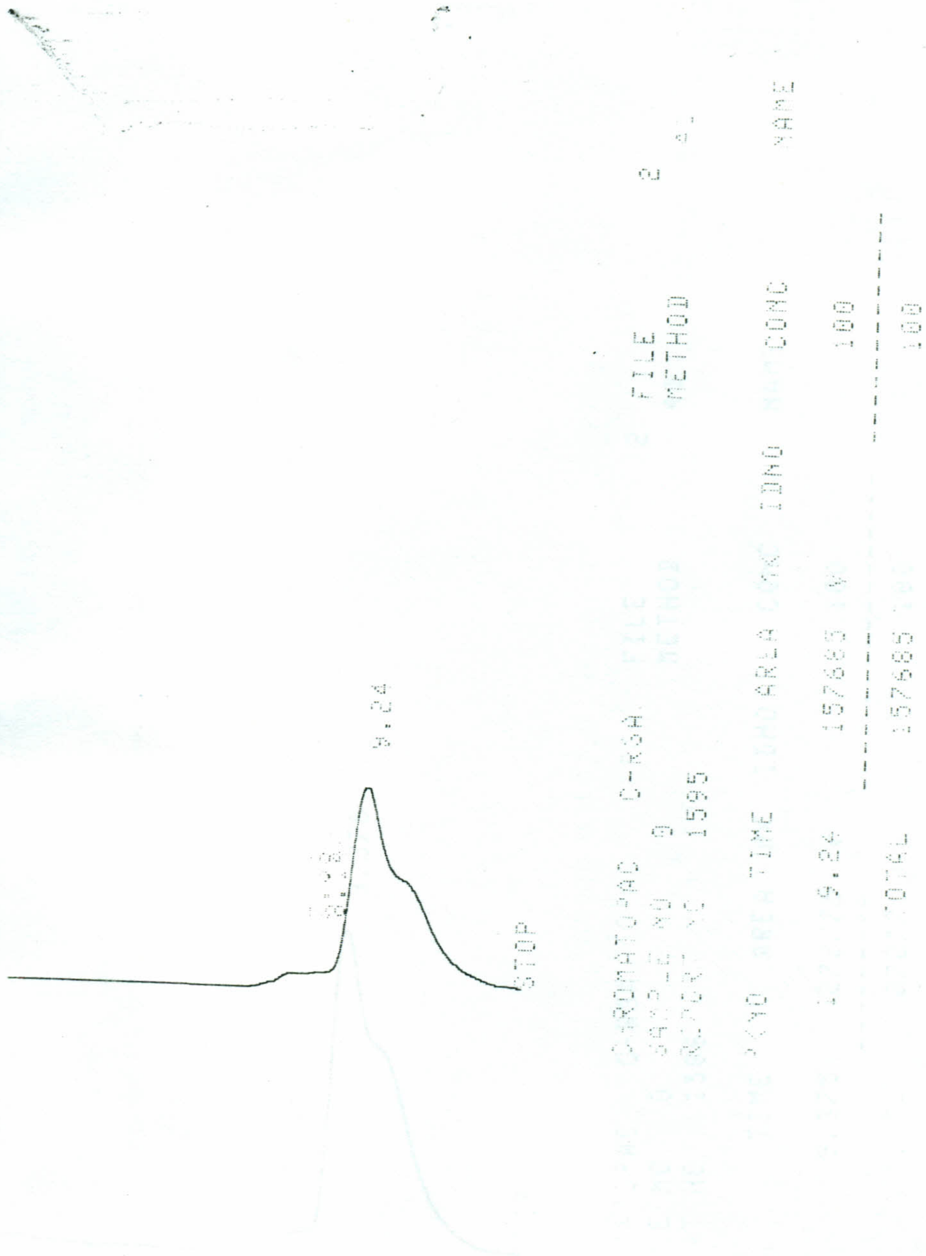


Figure E4. HPLC chromatogram for caffeine extract of Rukera coffee pulp sample.

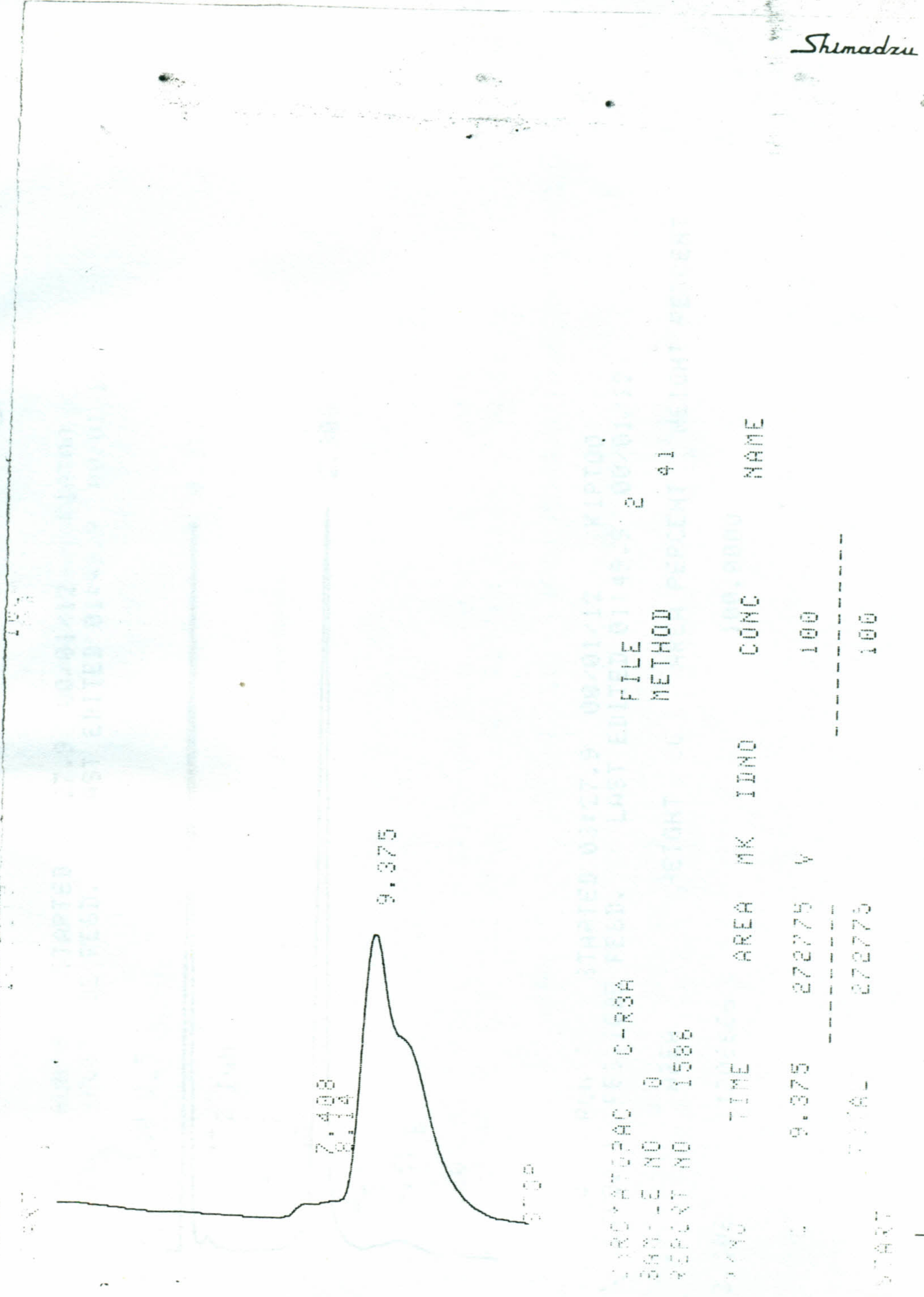


Figure E5. GC chromatogram for a chlorothalonil standard.

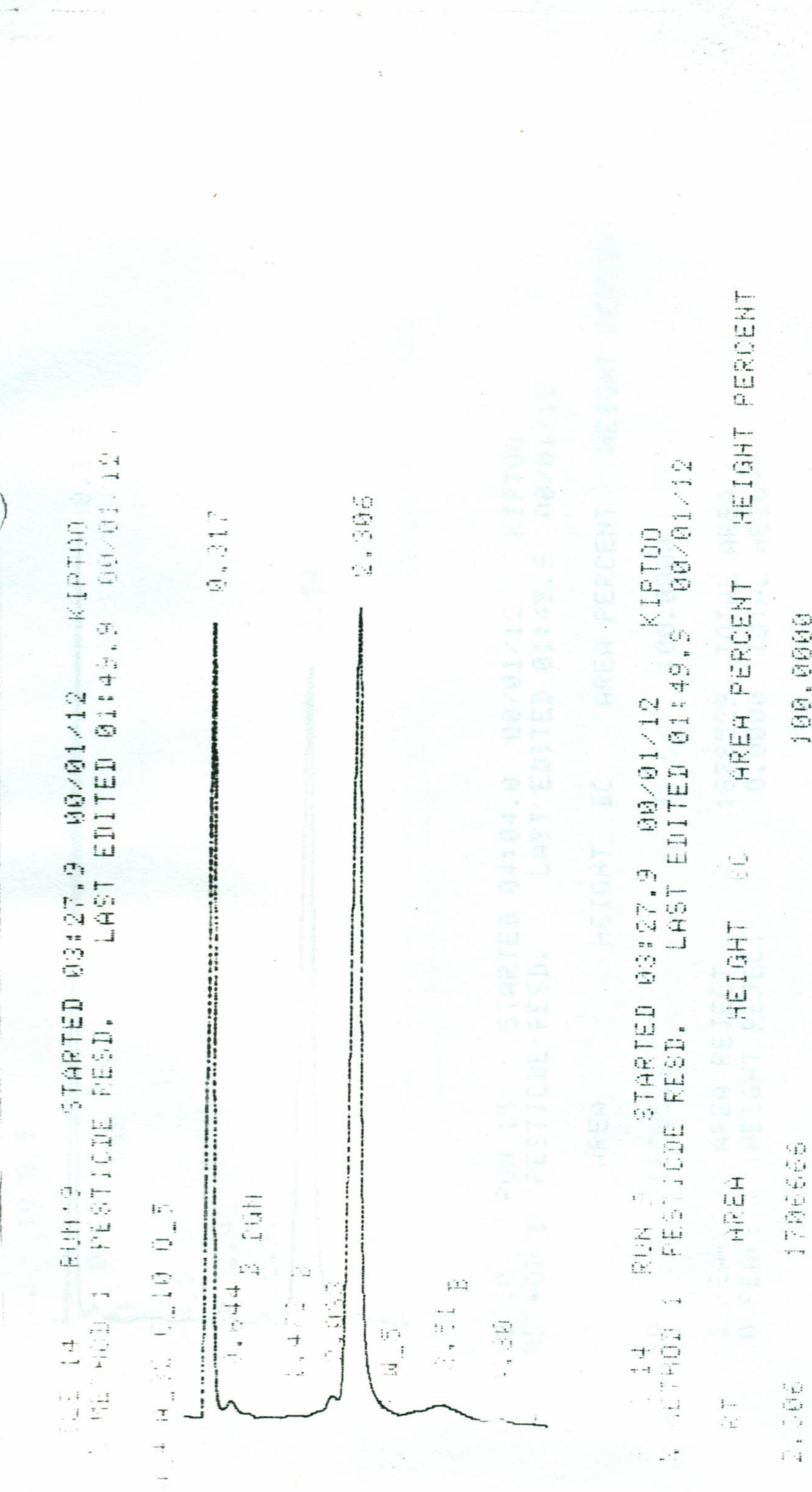
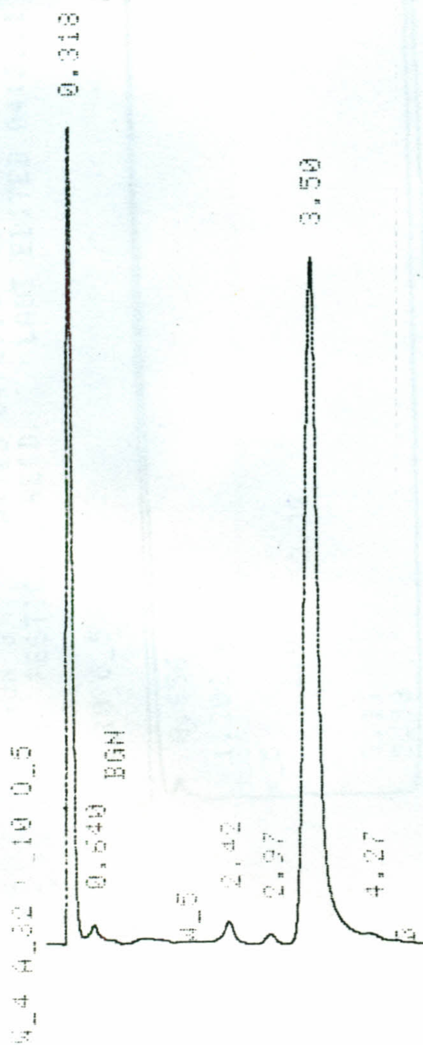


Figure E6. GC chromatogram for a fenitrothion standard.



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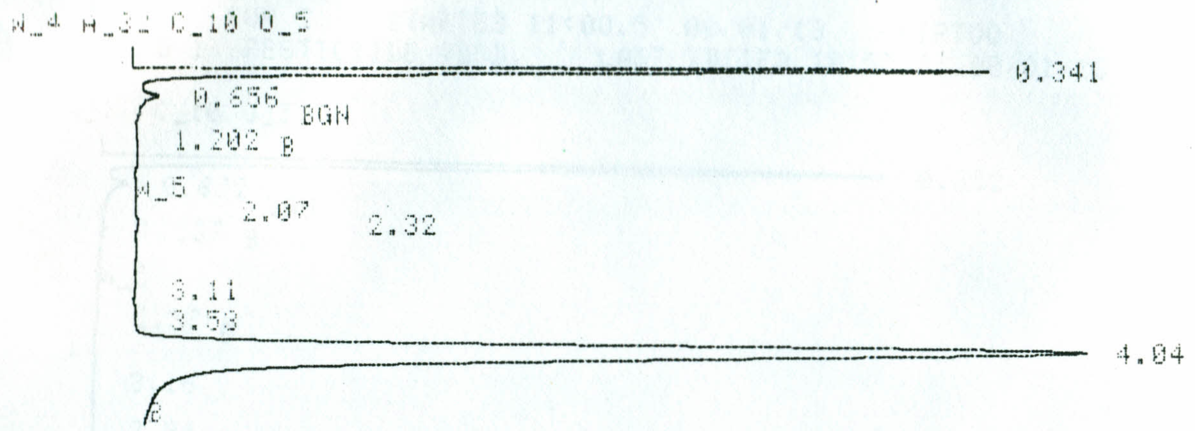
% METHOD 1 PESTICIDE RESD. LAST EDITED 04:32.1 00/01/12



FILE 3 RUN 3 STARTED 04:39.5 00/01/12 KIPTOO
% METHOD 1 PESTICIDE RESD. LAST EDITED 04:32.1 00/01/12

RT	AREA	HEIGHT	BC	AREA PERCENT	HEIGHT PERCENT
4.04	2720438			100.0000	

1 PEAK > AREA REJECT 2720438 TOTAL AREA
0 PEAKS > HEIGHT REJECT 0.0000 TOTAL HEIGHT

Figure E7. GC chromatogram for a chlorpyrifos standard.

Figure E8. GC chromatogram for a triadimefon standard.

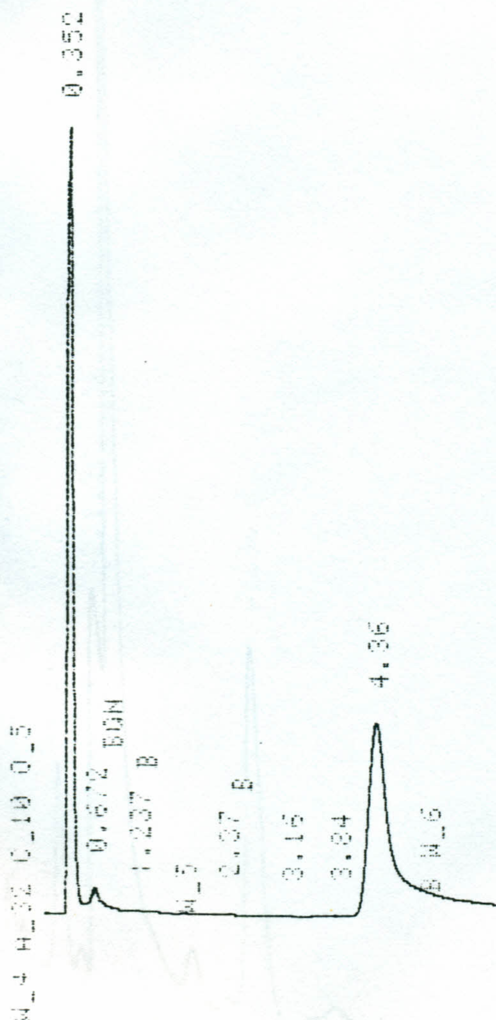


Figure E9. GC chromatogram for pesticide residues extracts of Ruiru Mills coffee pulp sample.

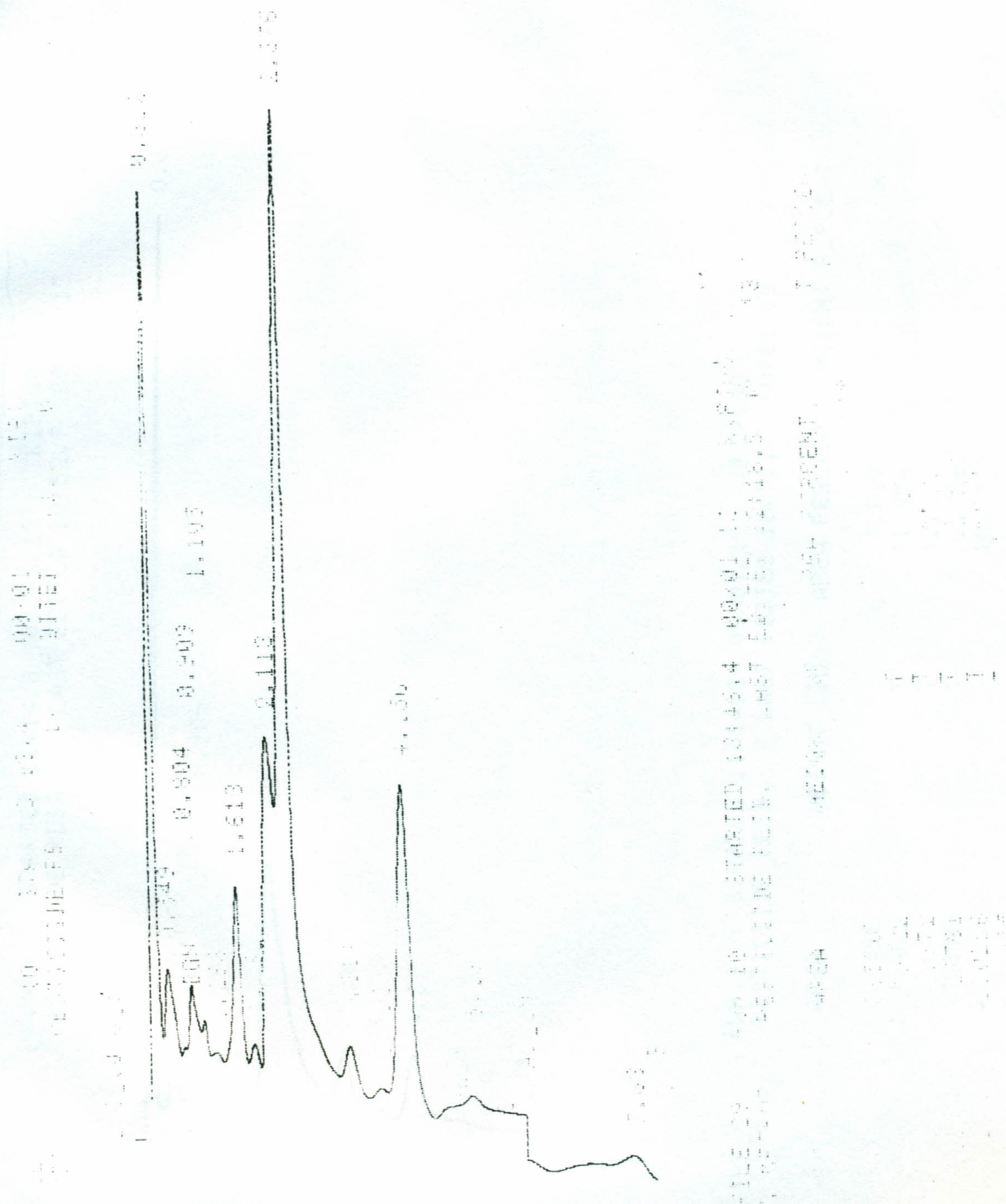


Figure E9. GC chromatogram for pesticide residues extracts of Tassia coffee pulp sample.

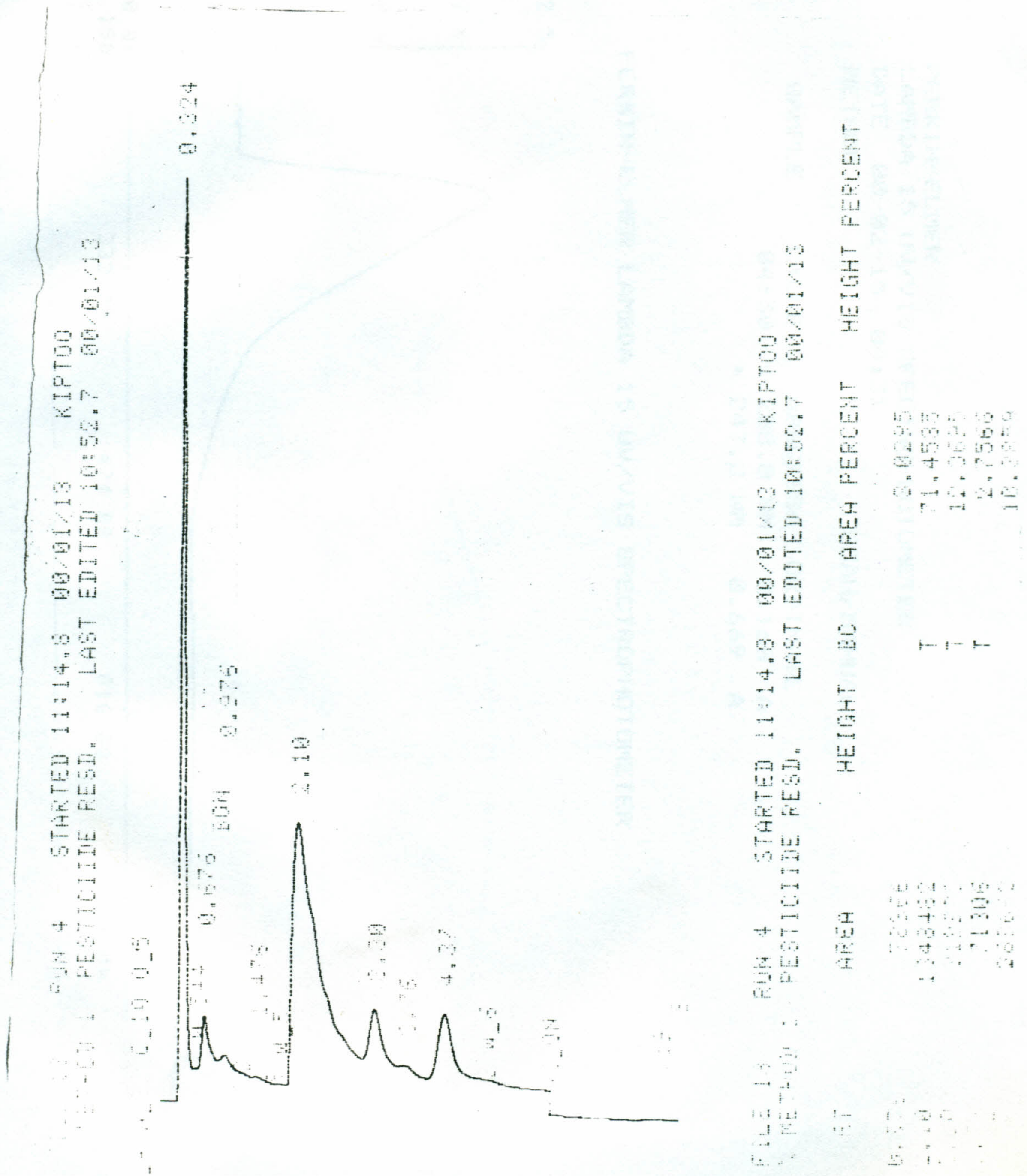


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Figure E10. Absorption spectrum for a tannin standard.

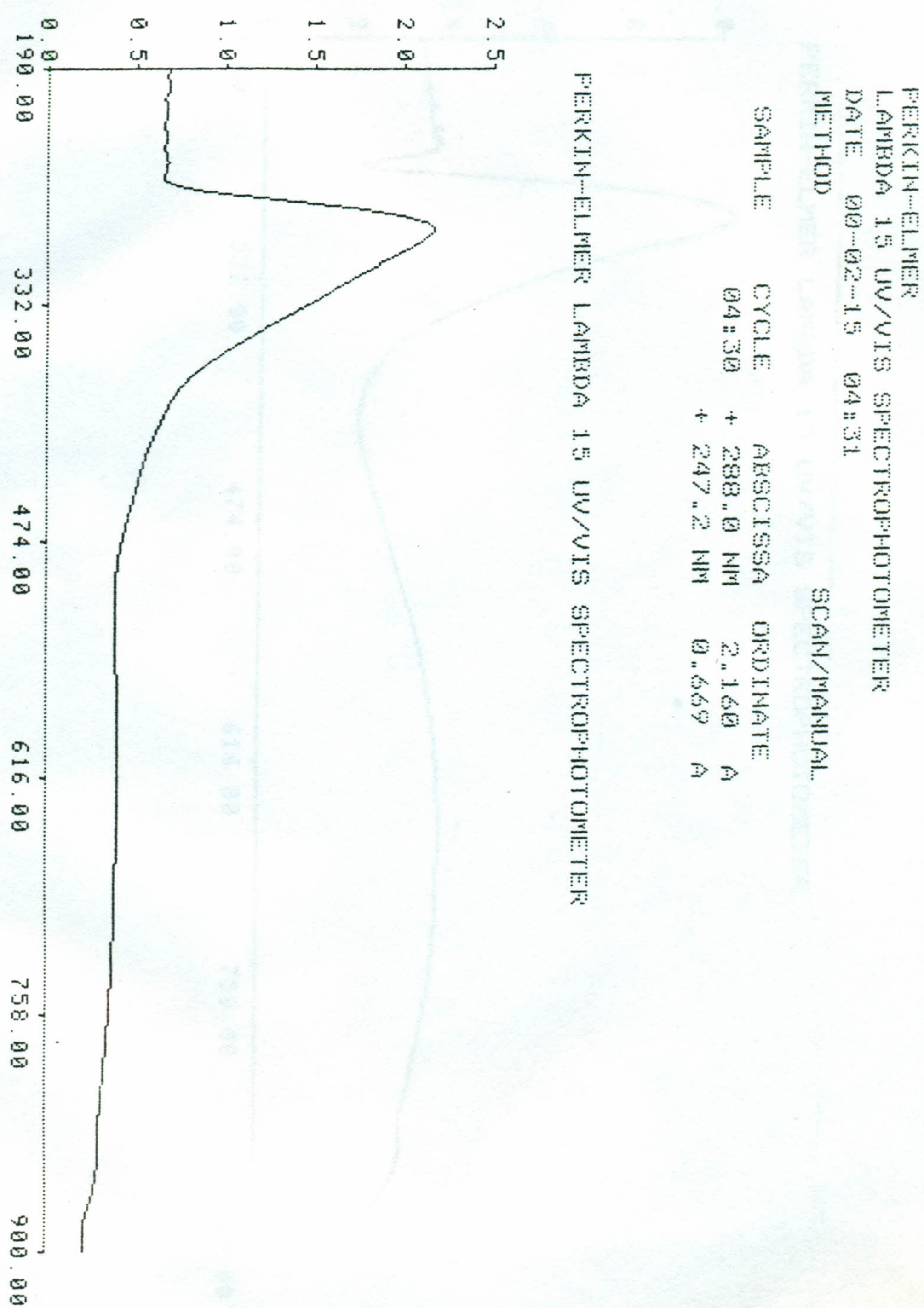

Figure E11. Absorption spectrum for tannin extracts of Rukera coffee pulp sample.