Article in press, volume 2 issue 1, 2012

EAPAS

East African Journal of Pure and Applied Science

East African Journal of Pure and Applied Science Vol. 2 (1): 73-103 (2012)

ASSESSMENT OF AMITRAZ RESIDUE IN MILK SAMPLES IN UASIN GISHU AND NANDI DISTRICTS

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ABSTRACT

Analysis of amitraz residue in milk samples, in Uasin Gishu and Nandi countiess has been done. The samples were randomly obtained from cows' farmers using amitraz as a spray and also milk vendors around Eldoret town. Levels of amitraz residue were compared with MRL and ADI levels given as 0.05ppm by WHO, FAO and EPA. GC/MS was used for qualitative analysis. GC/ECD was also used for quantitative analysis. Derivatization of the extractable of amitraz residue from spiked standards and milk samples was done with heptafluorobutyric anhydride, to enhance electron capture for greater sensitivity with ECD. Before spraying amitraz residue was low. After spraying there was a sharp increase, then sharp decrease followed by gradual decreases in concentration of amitraz residue. The average concentration of amitraz residue from milk samples ranged from 0.02 - 0.05 ppm with milk having amitraz residue falling below 0.22ppm. It was found out that 39.58% had an average of amitraz concentration below MRL. About 10.42% of samples had the range of 0.06-022 ppm, which is above the MRL, while about 8.33% had 0.05ppm MRL. The effect of optimising and derivatization gave good extraction conditions, which were 2 hours reflux times, 60 minutes water bath periods and 50^{o}C water bath temperatures. Detection limit using 3x s.d blank method was found to be 0.016 ppm which is below the MRL. The percentage recoveries of amitraz residue were above 80% the accepted value showing how effective extraction was. The butter fat content was found to fall in the range of 2.00 - 5.17% with most milk having butter fat content falling in the range of 2.50- 3.50%. A correlation between butter fat content and amitraz concentration was found to be 0.957 thus positive. The final result had an implication on the usage of amitraz product and its toxicity effect on the consumers. Hence risk mitigation had to be taken in account.

Key Words**:** Amitraz residue, milk, farmer's, Uasin Gishu, Nandi.

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 ISSN 2070 0903

INTRODUCTION

There is increasing social and political pressure to continuously monitor the quality of the environment. The impact of this calls for effective method for detection of various pollutants at very low levels in food, water and other important matrices (Holland and Makom, 1992). Environmental pollutants include pesticides, fertilizers, sanitation chemicals, preservatives and other chemicals. Pesticides are of economic benefit to man, but their potential for producing adverse health effects, resulting from low-level exposure dictates most of the world's environmental focus on them (European Commission, 2002).Pesticides are chemical substances intended for preventing, destroying, repelling or mitigating the effects of the pest. They are substances that kill or interfere in the life cycle of certain pests (Scientific encyclopaedia, 1996). They are classified as insecticides, fungicides, herbicides, and acaricides.

Cattle are at risk of infestation by three acaricides; ticks, mites and lice. (Shaw, 1969 and Technical Bulletin, 1996). The remedy to this problem is the use of acaricide to control them (Technical Bulletin, 1996). Different acaricides have been developed and include cypermethrine acaricides such as barricate, formamidine acaricide like amitraz among others. In recent times, amitraz has been the acaricide of choice because of its low residual toxicity (LD_{50} levels responsible for mortality of half test population of 800 mg/kg) and its broad spectrum , it kills ticks, mites, lice and keds (Griffith, 1975; Harrison and Palmer, 1981; Hill, 1987; Abed and Lihitte 1993; Technical Bulletin, 1996), in their eggs, moulding larval, nymph and adult stages of metamorphism (Harrison and Plamer, 1981, Technical Bulletin, 1996). Ticks are the most debilitating to cattle. These are parasites that withdraw up to 3ml of blood from its host when they engorge. They are amongst the most damaging (in terms of animal health) of all veterinary pests, producing wide losses conservatively estimated at 200 million sterling pounds annually, with 80% of the world wide cattle at risk (Shaw, 1969).

Milk is the most unique and ideal food for man. It meets the nutritional needs of the body better than any other food. It contains; proteins, carbohydrates, fats, minerals and vitamins in fairly soluble proportions. However, its quality has been affected much by many factors. Some of these are milk adulterants, of which amitraz, an active ingredient of an acaricide is. EPA, FAO and WHO have set MRL levels in food to be 0.05ppm, which will have no effect to the consumers. Above this value, food becomes dangerous to feed on.

Toxicity

Pesticides are supposed to be used without posing unreasonable risks to human health as well as the environment. But amitraz an as insecticide and acaricide has been shown to be toxic. From acute toxicity studies, amitraz is moderately toxic by dermal route (Toxicity category II) (EMEA, 2004). It is slightly toxic by oral and inhalation routes (Toxicity category III) and also non-irritating to the eyes and skin (Toxicity category IV) (EPA, 1996). Subchronic toxicity studies, show that higher doses caused reduced body weight gain and liver toxicity in mice (Sutton, 1973c and EPA, 1996). In dogs it affected the liver, kidney and central nervous system effect (EPA, 1996). In rabbits it caused skin reaction, anorexia, hyperglycaemia, degeneration of testes and effect on lymph nodes and various organs (Sutton, 1973(a)). Chronic study, using dogs resulted in central nervous system depression, increased blood glucose level and hypothermia. In carcinogenicity feeding study-using mice has shown lyphoreticular tumours in females, liver and lung tumours at highest dose levels studied (EPA, 1996). Based on these studies, EPA has classified amitraz as group C (possible human) carcinogen (EPA, 1996). Human volunteers who received a single oral dose of 0.25mg/kg ^{14}C–amitraz showed; drowsiness, disorientation, slurred speech, decreased pulse rate and blood

pressure, and other effects (Campbell and Needham 1984 c). Thus the envisaged application of results from this paper is expected to lead to the improvement of the quality of milk for consumers and advice farmers on dangers of incorrect usage of amitraz based pesticides as well as health matters of consuming unprocessed milk.

Regulatory history

Amitraz was registered as a technical pesticide grade in 1975 (EPA, 1996). EPA received an application for registration of an end-use product for apples and pears in 1976. In 1977 the pesticide went into special review called rebuttable presumption against registration or RPAR because it met the risk criteria for cancer effects (EPA, 1996). It was shown to cause cancerous tumours in mouse lymph systems (EPA, 1996 and EMEA, 2004). At the end of the RPAR process in 1979, EPA concluded that there was "weakly positive evidence" that amitraz is a possible human carcinogen (EPA, 1996). The Agency conditionally registered the pear use in 1980 since there were no alternatives for controlling pear psylla, but rejected the apple use since alternative pesticides were available (EPA, 1996). Parts of the conditional registration requirements were satisfied by submission of a new mouse cancer study, which the agency's cancer assessment group (CAG) evaluated in 1986. CAG classified amitraz as a Group C, possible human carcinogen, a classification that still stands (EPA, 1996). In 1986, EPA registered amitraz to control ticks on cattle and lice on hogs (EPA, 1996).

Amitraz identity

Human health risk assessment

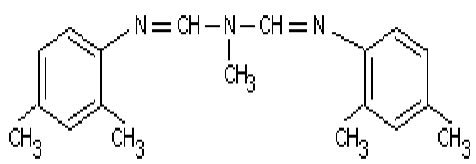
People may be exposed to residues of amitraz in pears and other foods. However, chronic exposure to amitraz residues in the diet is at a low level (only a small percent of the reference dose, RfD) (EPA, 1996). The concern is that amitraz has the potential to cause reproductive, developmental and neurological toxicity risks to the general population (FAO and WHO, 1980). Also the handlers applying amitraz to pear orchards, cotton fields and livestock on a long-term basis may be at risk for cancer effects (EPA, 1996).

Environmental fate assessment

Amitraz rapidly degrades in the environment to form two primary transformation products; BTS 27271 (*N-(2,4-dimethyl phenyl)-N-methyl formamidine*), BTS 27919 (*2,4-dimethyl formanilide*) and a secondary transformation product BTS 24868 (*2,4- dimethyl aniline*) (EPA, 1996). Because of its rapid degradation in the environment, amitraz is not expected to pose a concern for ground or surface waters. In contrast to amitraz, amitraz transformation products have been shown to be moderately persistent in aquatic and terrestrial environments and appear to be relatively immobile in soil column and field dissipation studies (EPA, 1996).

The chemical name of amitraz is N-methyl bis (2,4-xylyliminomethyl) amine and its structural formula is given in Figure 1 below.

Figure 1: Chemical structure of amitraz



Formulations

Amitraz is prepared in the laboratory by the reaction of 2,4-xylidine, ethyl orthoformate and methylamine (Harrison *et al.*, 1972)**.** It belongs to a class of compounds called amidines, which are

of the general form as shown in Figure 2 below (FAO and WHO, 1980).

The imine and amine moieties of amitraz determine its chemical behaviour and reactivity. It is available as emulsifiable concentrates 200 mg active ingredient per litre for crop-protection, 125 mg active ingredient per litre for animal use and as wettable powders containing 500 or 250 mg active ingredient per kg (FAO and WHO, 1980).

Figure 2: General form of amidine**,** where R_1, R_2 and R_3 = alkyl group and aryl group.

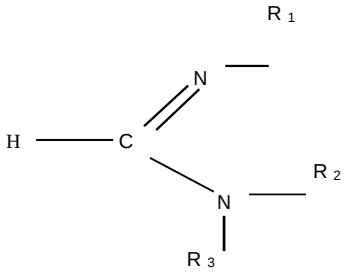
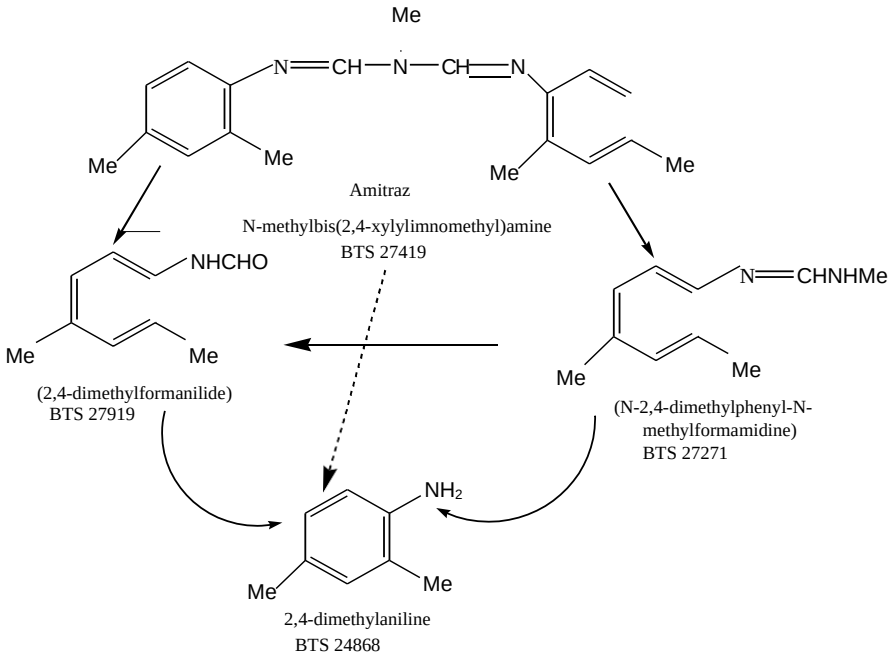


Figure. 3: Bacterial degradation of amitraz **(Adapted from Allock *et al.*, 1978)**



Metabolites

Amitraz is metabolised both by animal and plants to other related compounds. Studies have shown that amitraz degrades rapidly to yield predominantly *N-(2,4-dimethyl phenyl)-N-methyl formamidine* and a small amount of *2,4-dimethyl formanilidine* (FAO, 1984). Animals gave the same metabolites as plants with *N-(2,4-dimethyl phenyl)-N-methyl formamidine* and *2,4-dimethyl formanilidine* being common. This reaction involves oxidation reaction similar to those

occurring in the human body during glucose metabolism. Bacterial degradation of amitraz involves conversion of amitraz to *2,4-dimethyl aniline.* A slower pathway involves conversion of amitraz to *2,4-dimethyl aniline* via intermediates *N-(2,4- dimethyl phenyl)-N-methyl formamidine* and *2,4-dimethyl formanilidine* (Allock *et al.,* 1978) as summarized in Figure 3. This degradation is comparable to complex respiratory reactions involving NAD^+ in the human body. At pH greater than 11.5 bacteria are unable to degrade amitraz because the highly basic media affects bacteria processes (Backer and Woods, 1977). Amitraz is well absorbed, extensively metabolized, and rapidly excreted in urine; 62% within 24 hours and 73% within 96 hours. Residues concentrations are highest in liver, adrenal glands, and eyes (FAO & WHO, 1980).

Milk

Table 1: Composition of milk.

Constituents of milk from various mammals, average wt %							
Species	**Water**	**Fat**	**Protein**	**Lactose**	**Ash**	**Non-fat Solids**	**Total Solids**
Human	87.4	3.75	1.63	6.98	0.21	8.82	12.57
Cows							
Holstein	88.10	3.44	3.11	4.61	0.71	8.43	11.87
Freshian	88.00	3.50	3.25	4.62	0.75	8.60	12.43
Ayrshire	87.40	3.93	3.47	4.48	0.73	8.68	12.61
Brown Swiss	87.30	3.97	3.37	4.63	0.72	8.72	12.69
Guernsey	86.40	4.50	3.60	4.79	0.75	9.14	13.64
Jersey	85.60	5.15	3.70	4.75	0.74	9.19	14.34
Goat	87.00	4.25	3.52	4.27	0.86	8.65	12.90
Buffalo (India)	82.76	7.38	3.60	5.48	0.78	9.86	17.24
Camel	87.61	5.38	2.98	3.26	0.70	6.94	12.32
Mare	89.04	1.59	2.69	6.14	0.51	9.34	10.93
Ass	89.03	2.53	2.01	6.07	0.41	8.49	11.02
Reindeer	63.30	22.46	10.30	2.50	1.44	14.24	36.70

Milk is lacteal secretion practically free from colostrums. The nutritive value of milk depends on its composition. At the time of secretion, milk contains two liquid phases, fat (organic) and water (aqueous) between which are partioned at least forty chemical compounds (Henry and Newlander, 1977). Dissolved in the fat or held at fat globule surface are numerous compounds such as; proteins (casein and albumin), phospholipids, sterols, carotenoids and fat-soluble vitamins, while in aqueous phase consist of lactose (milk sugar), water-soluble vitamins and some of the minerals (Kon and Gowie, 1976). Fat ranges from 2.5 to 8.0 % while water ranges from 82 to 90 % (Freeman, 1959). This difference in composition of milk determines the distribution of organic and inorganic residue in it. Organic compounds tend to concentrate in fat layer while inorganic compounds concentrate in aqueous layer. Three physical states; solution, emulsion and colloidal (suspension) have a very intimate association such that changes in one of the states will affect one or both of the others. Although some minor constituents may be present in both fat and aqueous phases, it is convenient to consider milk as a mixture of water, fatty and non-fatty constituents (Kirk – Othmer, 1981).

Milk matrices

Physical and chemical action alters the intimate association of the physical state present, and the distribution of compound in this state. Milk has organic and aqueous phase. These constituents interact physically or chemically with adulterants, preservatives, pesticide residues and sanitation chemicals (Robertson, 1958). The interactions are quite complex and determine the distribution of these adulterants within the milk matrix. Storing of milk results in clamping up of individual fat globules, causing them to merge and form larger globules which rise to the cream layer, giving the top part of the milk a yellow colour (Henry and Newlander, 1977). This action can redistribute the organic substances in milk and concentrate them in the fatty layer. Stirring reduces viscosity of milk due to reduced fat clamping. High fat content lowers the surface tension while pasteurisation increases it (Anon, 1977). Changes in surface tension affect distribution of surface-active component like fats and proteins. Viscosity, surface tension and fat clamping are important factors to consider when sampling for the analysis of organic adulterants in milk (Bradfield, 1957). Bacteria are present in the milk from milk glands, ducts and also from external sources, such as utensils, stable air, animal skin, and from the milkier (Breed, 1975). They metabolise organic substances leading to the formation of new compounds that further complicates distribution of adulterants. Lactic acid is produced, which lowers the pH and causes the equilibrium established by amphoteric substances like amino acid to shift. Some of the adulterant detected in milk include; antibiotics used for treatment of mastitis, hypochlorites and chloroamines used for cleaning and disinfecting of milk utensils, organochlorine and organophosphate pesticides residue (Henry and Newlander, 1977; Mc Dougall et al., 1979). For accurate chemical analysis of constituent and adulterants, the most effective way of obtaining a representative sample for fat test is to mix milk by pouring it from one pail to another. At least three times to redistribute the fat (Bernard, 1975) is achieved, with care not subjecting the milk condition which are conducive to churning or freezing during cooling and storage to avoid creaming (Bradfield, 1957 and AOAC, 1975).

Sampling and validity of samples

A valid sample is one that is drawn randomly from a population, which all other samples that may be drawn have equal chance of being drawn. This gives representative information about population from which they are drawn (Danielle and Terrell, 1975). In heterogeneous populations, the method of sampling becomes important because it estimates one or more population characteristics (parameters). The goodness of this is that the sample will depend on how well it will estimate population parameters of interest (Hamilton, 1968).

Stratified random sampling

In this method, observational unit is also the sampling unit. Population of interest is subdivided into sub-populations (strata) based on a known variable that is associated with the measurement made on observation unit. This should be homogeneous relative to the measurement of interest which provides estimators that have smaller variances. Hence reduces the cost and creates greater administrative convenience. Milk samplers have used this method creating their strata based on variables ranging from location, climate, and type of pasture and foodstuff (Mc Dougall et al., 1979). This method was used in this research basing on the locations.

Analytical techniques

Analytical techniques available for amitraz determination in milk include; GC, UV, IR and HPLC. Others are TCD, FID, ECD and MS. IR methods are subject to interference by C=N bond of the metabolites and UV methods are less accurate (Willard et al., 1986). HPLC with UV detection needs larger volumes of solvent and high detection limit makes it less popular. Preparation of suitable solvents system for separation is also time consuming (Willard et al., 1986). GC methods are

most sensitive for amitraz analysis with ECD or FID. When ECD is used, derivatization of amitraz and its metabolites are done with heptaflourobutyric anhydride to produce electron-capturing species which detector responds to (Novotny, 1978). With FID no derivatization is done. GC/MS is used for confirmation of peaks of interest. While GC/ECD is used for quantification of amitraz in milk samples.

Qualitative analysis

Qualitative analysis of a component is its retention time under a given condition. Chromatography has a Quantitative analysis.

This is done by comparing of either the height or area as parameters of the analyte peak with that of one or more standards. If conditions are properly controlled these parameter vary linearly with concentrations (Novotny, 1978). Small number of data points as compared to single spectrum, of which spectral data is more accurate. But chromatography recognizes the present or absences of components of mixture containing a limited number of species, whose identities are known (Lee et al., 1984). Chromatograms provide a sure evidence of presence or absences of certain compounds by comparing the t_R (retention time) of standard and samples run under similar conditions in the chromatograms.

Based on peak area

Most modern equipments have digital integrators while old ones had ball-and disc integrators or in their absence, manual estimators had to be made. Methods of estimation include (for systematic peaks with reasonable widths) multiplying height of peak by its width at ½ peak height or using planimeter, determining weight of peak relative to weight of a known area of a recorder paper. Of all this electronic integration, which incorporates computer software, is the most accurate (Willard *et al.,* 1986).

MATERIALS AND METHODS

Sampling and Sample Preparation

Assessment of residue in samples is quite sensitive because concentrations are usually in range of ppm (very trace amount). Care should be taken into the account to avoid contamination that would cause erroneous results. Dairy farmers were identified in Nandi district in Kapsabet, Itigo, Kaptumo and kapkangani and randomly selected for the study. Also milk vendors were identified in Uasin Gishu district in Eldoret and its surroundings and randomly selected for study. Milk samples were collected in pre-washed 0.5 l polyethene bottles once daily for one day before spraying and seven days after spraying also once from different milk vendors. The milk samples were then bagged under ice in a polythene bag while still fresh. The fresh milk samples were then transported immediately to the laboratory for extraction and analysis GC/Ms and GC/ECD.

Analar reagents used were: Hydrochloric acid, 37%, Ethyl acetate, Sodium hydroxide pellets, Anhydrous Sodium sulphate (Na_2SO_4), n-hexane, 5 M Ammonium solution, Gerber's acid (sulphuric acid s.p. 1.82 to 1.83), Amyl alcohol (isopropanol s.p. 0.814 to 0.816), Dichloromethane and acetone, Amitraz was a technical grade, Heptafluorobutyric anhydride was a derivatization grade.

The instruments were: Explosion proof centrifuge, Top pan balance or Analytical balance, Gerber centrifuge, Gas chromatography/Electron capture detector and Gas chromatography/Mass spectrometer.

Amitraz stock solution (125 ppm), 10M NaOH, 2M HCl and 5% Ammonium Solution was prepared. Calibrating standard solutions were made by serial dilution to form 0.5, 1.0 and 1.5 ppm. Optimisation of the derivatization reaction conditions was done for reflux period 1hr, 2hr and 3hr; water bath period 40, 60, 70, and 80 minutes; and Water bath temperatures 40 ^oC, 50 ^oC and 60 ^oC. Amitraz residue was extracted using 37% HCl then clean - up with ethyl acetate and 10M NaOH. Derivatization reaction was done with heptafluorobutyric anhydride, 5 % ammonium solution and dried with

anhydrous sodium sulphate (Na_2SO_4). Samples were injected into the GC/MS and GC/ECD for analysis. Fat content analysis was done in butyrometers (Methodology adapted from Ministry of Public, Welfare and Sport, 1996).

RESULTS AND DISCUSSION

Results

The results for mean peak areas of derivative formed from reflux times (2 hrs), water bath periods (60 minutes) and water bath temperatures (50 °C), with the largest mean peak areas signified the conditions at which maximum amount of derivative (N-heptaflourobutyrl-2,4-xylidine abbreviated as NH 2,4X) was formed and are summarized in Figure 4.

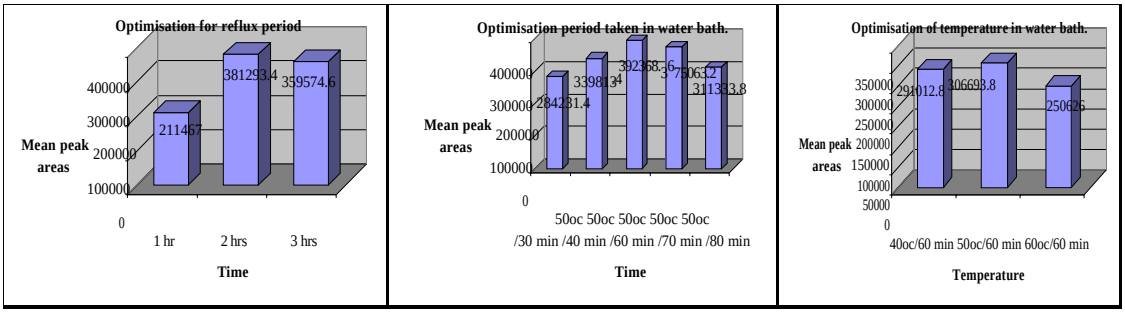


Fig. 4 Bar graph for optimisation of reactions

Reproducibility of data.

Split mode RSD%

- 16.933 for 0.5 ppm
- 35.217 for 1.0 ppm
- 22.243 for 1.5 ppm

Data not reproducible

RSD >10%

Split mode not used.

This data was not reproducible since relative std deviation is above 10% hence split mode was not used in this research.

Limit of detection.

Detection limit was 0.016 ppm using 3x s.d blank method. This was below MRL (0.05ppm) hence suitable and satisfactory analytical technique used.

Splitless mode RSD%

- 0.499 for 0.5 ppm
- 0.550 for 1.0 ppm
- 0.366 for 1.5 ppm

Data reproducible

RSD < 10%

Splitles mode used.

This data was reproducible since relative std deviation is below 10% hence splitless mode was used in this research.

Percentage recovery

At 0.5ppm Percentage recovery was 100%.

At 1.0ppm Percentage recovery was 99%.

At 1.5ppm Percentage recovery was 100%.

At 0.5ppm Percentage recovery was 83%.

At 1.0ppm Percentage recovery was 96%.

At 1.5ppm Percentage recovery was 100%.

Milk samples

These percentage recoveries were above the accepted value 80%. showing how effective extraction of amitraz residue in milk samples (accurate methodology used).

TABLE 2*:* Milk samples extracted & analysed 1 day before & 7 days after spraying

Select farmer	**Before spray**	**After spraying**						
		Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**
A	ac 0.02 bf 4.97	ac 0.22 bf 5.00	ac 0.07 bf 4.93	ac 0.05 bf 5.15	ac 0.04 bf 5.10	ac 0.03 bf 4.99	ac 0.03 bf 4.96	ac 0.02 bf 4.94
B	ac 0.01 bf 3.93	ac 0.12 bf 3.90	ac 0.04 bf 3.95	ac 0.03 bf 4.15	ac 0.02 bf 3.96	ac 0.02 bf 3.94	ac 0.01 bf 3.90	ac 0.01 bf 3.87
C	ac 0.01 bf 4.40	ac 0.17 bf 4.50	ac 0.05 bf 4.55	ac 0.04 bf 4.52	ac 0.03 bf 4.48	ac 0.02 bf 4.53	ac 0.02 bf 4.60	ac 0.01 bf 4.50
D	ac + bf 3.48	ac 0.10 bf 3.50	ac 0.03 bf 3.58	ac 0.02 bf 3.65	ac 0.02 bf 3.55	ac 0.01 bf 3.50	ac 0.01 bf 3.45	ac + bf 3.50

Mean amitraz concentration & butterfat for selected farmers A,B,C& D

Mean	**Before spray**	**After spraying**						
		Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**
amitraz butterfat	ac 0.0133 bf 4.195	ac 0.1525 bf 4.225	ac 0.0475 bf 4.253	ac 0.0475 bf 4.253	ac 0.0275 bf 4.285	ac 0.02 bf 4.240	ac 0.0175 bf 4.228	ac 0.0133 bf 4.203

Mean amitraz & butterfat for individual farmer A,B,C and D cows

Mean	**Farmer A (Jersey cow)**	**Farmer B (Ayrshire cow)**	**Farmer C (Guernsey cow)**	**Farmer D (Freshian cow)**
amitraz butterfat	ac 0.06 bf 5.005	ac 0.0325 bf 3.95	ac 0.04375 bf 4.51	ac 0.0316667 bf 3.52625

Mean amitraz & butterfat for farmer A,B,C and D cows	
amitraz butterfat	ac 0.0419792 bf 4.2478125

Select farmer A= Kapsabet, B= Itigo, C= Kaptumo, and D= Kapkangani (Nandi District)

Note: ac means amitraz concentration.

bf means butter fat

+ means amitraz was detected but not quantified

TABLE 3: Milk samples extracted analysed in Eldoret town & its surrounding

Study area, sampled from milk vendors,Uasin Gishu	**Amitraz concentration and butterfat content**			**Mean**
Beta farm	a c - b f 3.50	0.02 2.50	0.04 3.80	0.0200 3.2667
Chep	a c - b f 4.80	- 5.20	- 4.50	- 4.8333
Elgon view	a c 0.22 b f 4.50	0.04 3.58	0.05 4.00	0.0967 4.0267
Hawaii	a c 0.04 b f 2.90	+ 1.90	0.03 2.00	0.0233 2.2667
Huruma	a c 0.10 b f 4.30	0.04 2.55	0.05 3.77	0.0633 3.5400
Junction (Iten/chep)	a c - b f 3.50	- 4.20	- 3.00	- 3.5667
Kamukunji	a c 0.02	-	+	0.0067

	b f 2.25	2.10	1.90	2.0833
Kapsowar	a c 0.04 b f 3.65	+ 3.25	- 3.50	0.0133 3.4667
Kidiwa	a c 0.03 b f 2.56	- 1.90	0.03 2.11	0.0200 2.1900
Langas	a c 0.04 b f 3.50	0.05 3.66	0.03 2.88	0.0400 3.3467
Mail nne	a c - b f 3.56	0.03 3.40	+ 3.88	0.0100 3.6133
Munyaka	a c - b f 2.50	- 2.77	- 2.31	- 2.5267
Road block	a c 0.06 b f 4.50	+ 3.20	0.03 3.45	0.0300 3.7167
Town centre	a c 0.05 b f 4.53	0.06 5.17	- 4.32	0.0367 4.6733
West	a c 0.04 b f 3.82	0.03 3.50	0.04 3.60	0.0367 3.6400
West Indies	a c 0.07 b f 4.35	0.02 4.54	0.03 4.25	0.0400 4.3800
Overall mean Amitraz concentration and butterfat content				a c 0.0273 b f 3.4398

Note: ac means amitraz concentration.

bf means butter fat.

+ means amitraz was detected but not quantified

- means amitraz was not detected

Average correlation between butter fat content and amitraz concentration was found to be 0.957. Most milk had butter fat content falling in the range of 2.50 - 3.50. This shows that an increase in butter fat increases amitraz residue by nearly 95 %.

Discussion

Optimization of reaction

An attempt was made to maximise product formation in the shortest time possible while utilising the least amount of reagents and the result was in lowering the cost of carrying out this research. This was done by optimising one condition while holding others, and repeating the procedure until all conditions had been optimised (univariate optimisation). The results for mean peak areas of derivative formed from reflux times (2 hrs), water bath periods (60 minutes) and water bath temperatures (50 ^{o}C), with the largest mean peak areas signified the conditions at which maximum amount of derivative (N-heptaflourobutyrl-2,4-xylidine abbreviated as NH 2,4X) was formed.

Confirmation of peak by derivative N-heptaflourobutyrl-2,4-xylidine (NH2,4X)

The derivative N-heptaflourobutyrl-2,4-Xylidine (NH 2,4X) was formed by (I) hydrolysis of amitraz with conc. HCl (37%) then (II) warming with derivatizing agent (heptaflourobutyric anhydride) in water bath thermostated at 50 ^{o}C for 1hr. The reaction is given in Fig 5.below.

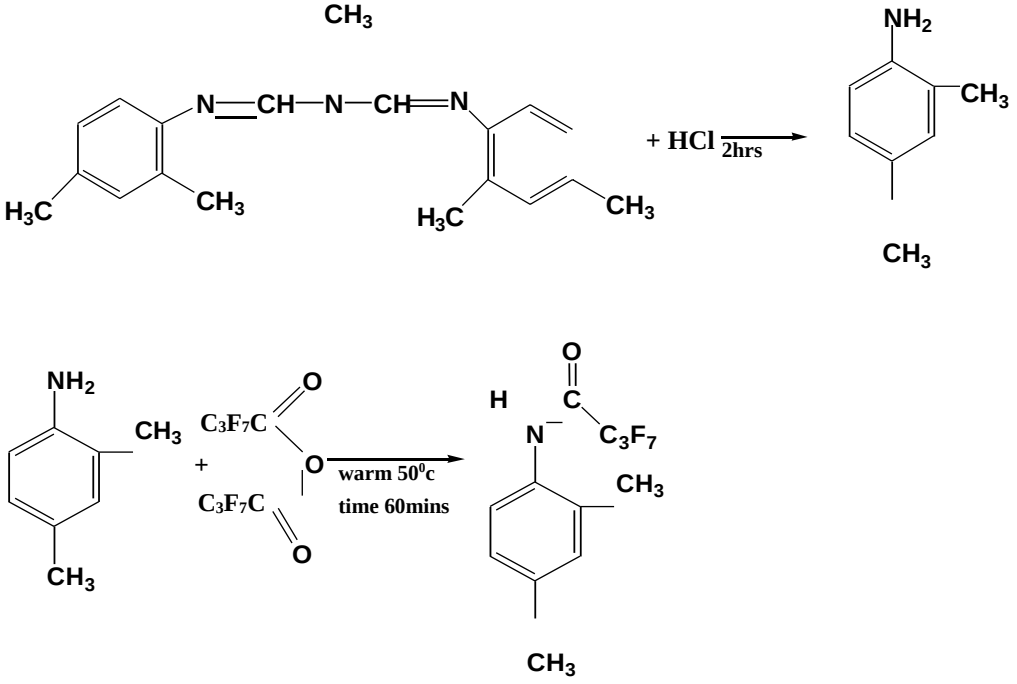
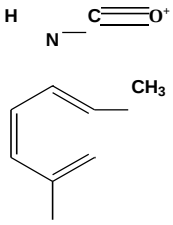
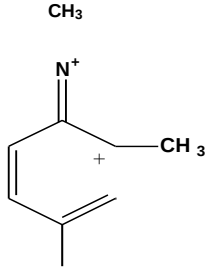


Fig. 5: Formation of N-heptaflourobutyrl-2,4-xylidine (NH2,4X).

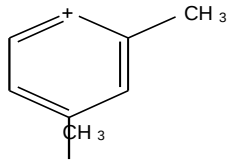
From mass spectroscopy the fragmentation ion of NH 2,4X ionised by electron impact (EI). The ions m/e = 148, 120, 105 and 317 were the most abundant because of delocalisation of charge due to conjugation and resonance effects. The structures of the ions are given in Figure 6. M/e=148 was the most abundant due to its stability. A computer library was used to search and match



most stable ion,thus most abundant (m/e=148)

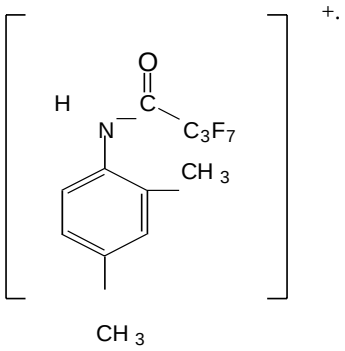


(m /e=120)



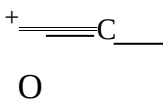
/ e = 1 0 5

Reproducibility depends on mode of injection, that is split ratio or splitless mode. From the spiked standards, the mean peak areas were given by GC/ECD and standard deviation (S.D) and relative standard deviation (R.S.D.) were calculated. If (R.S.D.) values are lower than 10% (<10%) it shows that the data is reproducible, facility giving the probable identity of NH 2,4X that is a value of matches F: 1000 means a perfect match, which is rare (usually it is less than 1000). Direct comparison of the two showed close similarities, thus confirming the presence of NH 2.4X (positive). The NH 2,4X gave a peak at a retention time of 2.27 - 2.28 minutes in GC.



(m/e = 317)

$C_3F_7^+$ (m/e=169)



m/e = 197, least stable ion thus least abundant

Fig. 6: Fragmentation ion of NH 2,4X

reproducibility of data

while for (R.S.D.) greater than 10% (>10%) then data is not reproducible. Splitless mode tends to have lower values as compared to split mode. Therefore, for reproducibility to occur, split mode will need high concentration of sample. But in this study trace analysis (range of picogram) was dealt with. Thus splitless mode was used as it allowed all samples into the column while most of the solvent was pushed out of the column. Unlike in split mode where only part of the sample goes into the column

according to selected ratio. The data from split mode was not reproducible since relative standard deviation s were above 10% hence was not used in this study, while **Limit of detection**

data from splitless mode were reproducible since relative standard deviations were below 10% hence was used in this research.

In general limit of detection of analyst is described as a concentration of analyte which gives an instrumental signals significantly different from the blank or background signal (Miller and Miller, 1993) and is very important because it shows the relevant of analytical techniques to be used. Limit of detection is the analyte concentration giving a signal equal to the

blank signal $Y_{B,}$ plus three standard deviation of the blank $S_{B.}$

$Y = Y_B + 3 S_B$ (miller and miller, 1993). Since analysis done is with a MRL of 0.05ppm, then a detection limit lower than 0.05 ppm was required. The limit of detection 0.016 was below MRL (0.05ppm) hence suitable and satisfactory analytical technique used.

Percentage recovery

The percentage recovery was obtained by calculating the actual concentration of the spiked standard solutions and dividing by the corresponding spiked levels, that is, Percentage recovery = (actual concentration/spiked standard concentration) x 100. The percentage recovery shows that the method of extraction and derivatization is satisfactory when percentage is high, that is 100 %, meaning that all amitraz was

extracted and derivatized. Lower percentage values means that either extraction or derivatization or both were not completed and the accepted recovery percentage should be above 80 % for it to hold. For this study all the values for percentage recovery were above 80 % for concentrations between 0.5 ppm and 1.5 ppm. and high percentage recoveries showed how effective extraction of amitraz residue in milk samples (accurate methodology).

Spiked standards and milk samples

Calibration was obtained for each set of data and lines of best fit plotted using computer software (Excel). Using lines of best fit, concentration of spiked standard solutions and milk samples were calculated and GC/ECD gave chromatograms of retention times for peak areas. The mean peak areas were then used to calculate the actual concentrations. Using the line of best fit by Excel, the concentration of spiked standard

(0.5, 1.0, and 1.5) ppm verses their respective mean peak areas were plotted. The derivatized amitraz standard solution was run concurrently with milk samples, which were also extracted and analysed for amitraz residue, one day before spraying, and seven days after spraying. This enabled one to understand the behaviour of amitraz in milk in relation with time length (persistence of amitraz residue).

TABLE* 4: *Persistence of amitraz residue in milk samples

Selected farmer	**Before spray**	**After spray**						
		Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**
A	ac. 0.02	ac.0.22	ac.0.07	ac.0.05	ac.0.04	Ac 0.03	ac.0.03	ac.0.02
	Detected in milk		31.81%	22.73%	18.18%	13.64%	13.64%	9.09%
B	ac.0.01	ac. 0.12	ac. 0.04	ac. 0.03	ac.0.02	ac.0.02	ac.0.01	ac. 0.01
	Detected in milk		33.33%	25.00%	16.67%	16.67%	8.33%	8.33%
C	ac. 0.01	ac.0.17	ac. 0.05	ac.0.04	ac. 0.03	ac. 0.02	ac. 0.02	ac 0.01
	Detected in milk		29.41%	23.51%	17.65%	11.76%	11.76%	5.88%
D	+	ac.0.10	ac.0.03	ac.0.02	ac.0.02	ac.0.01	ac. 0.01	+
	Detected in milk		30%	20%	20%	10%	10%	∞
Mean amitraz concentrations for selected farmers A ,B ,C and D								
Mean amitraz conc.	0.0133	0.1525	0.0475	0.0350	0.0275	0.0200	0.0175	0.0133
	Detected in milk		31.1475	19.6721	18.0328	13.1448	11.4754	8.7213

Selected farmer A; Kapsabet, B; Itigo, C; Kaptumo, and D; Kapkangani (Nandi District)

Note: ac means amitraz concentration.

bf means butter fat.

Detected in milk means amitraz detected in milk in terms of percentage.

+ means amitraz residue was detected but not quantified.

∞ means amitraz residue not determinable in terms of percentage.

Observed information:
Both farmers A and B had programmed the drinking of water by the cows while farmers C and D had water constantly available to the cows

TABLE 5: Persistence of mean amitraz residue in milk for farmer A, B C, & D

Farm	Before spray	After spray						
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
A	0.02	0.22	0.07	0.05	0.04	0.03	0.03	0.02
B	0.01	0.12	0.04	0.03	0.02	0.02	0.01	0.01
C	0.01	0.17	0.05	0.04	0.03	0.02	0.02	0.01
D	+	0.1	0.03	0.02	0.02	0.01	0.01	+
Mean	0.0133	0.1525	0.0475	0.035	0.0275	0.02	0.0175	0.0133

Note + mean amitraz residue was detected but not quantified below limit of detection.

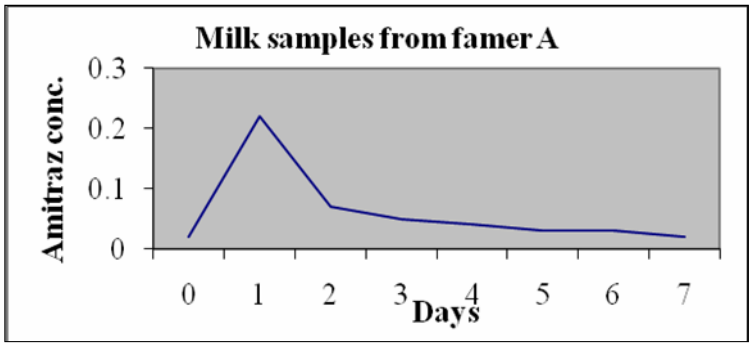


Fig.7: Persistence of amitraz residue in milk samples for farmer A.

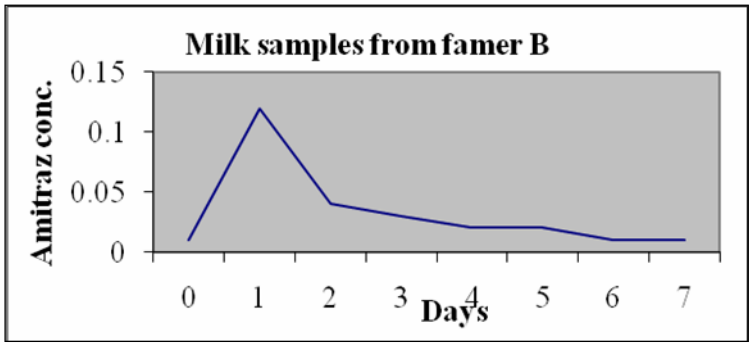


Fig. 8: Persistence of amitraz residue in milk samples for farmer B.

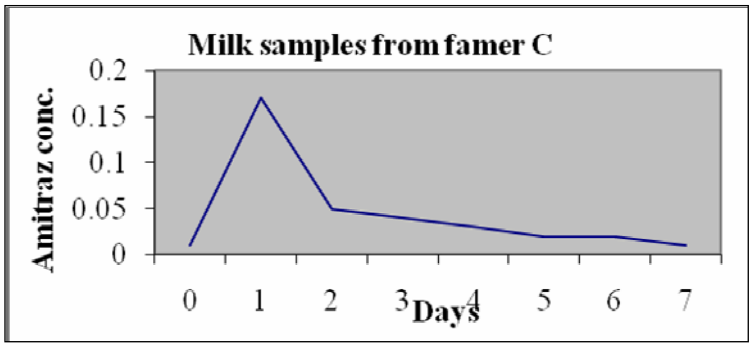


Fig. 9: Persistence of amitraz residue in milk samples for farmer C.

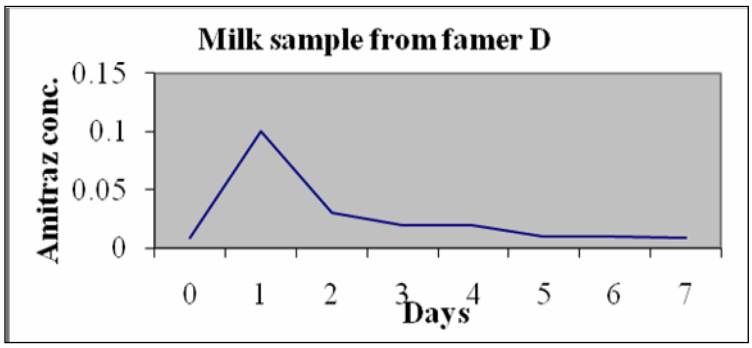


Fig. 10: Persistence of amitraz residue in milk samples for farmer D.

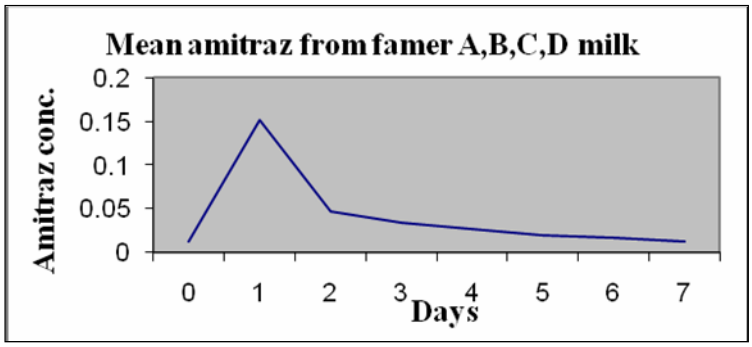


Fig. 11: Persistence of mean amitraz residue in milk for farmer A, B, C and D.

Variation of amitraz concentration in milk samples

There was a sharp increase in amitraz residue between days 1 and 2 followed by a sharp decrease between days 2 and 3, then a gradual decrease as the number of days increases to 7. This is shown in Fig. 7 to 10. This pattern is observed in all the milk samples for all the farmers A, B, C and D. Milk sampled on the 1st day after spraying had the highest amitraz residue (above MRL), hence the most toxic (0.22, 0.12,
0.17 and 0.1) ppm see Table 5. The only milk which had amitraz residue above MRL was on day 2 for farmer A (0.07ppm) while for farmer C had 0.05ppm, which is MRL. The rest of milk samples had amitraz residue below MR, see Tables 4 and 5. For farmer D, before spraying and day 7, amitraz residue was detected but not quantified because it was below the detection limit of ECD. However since the value was below MRL, it could not cause any risk on human health. See Tables 4 and 5. Both farmers A and B had programmed the drinking of water by their cows while farmers C and D had water constantly around the cows. The effect of this had shown some differences in decrease of amitraz residues between days 1 and 2 after spraying. For farmers A and B there was a decrease to 31.81% and 33.33%, respectively (a mean of 32.57%) while for farmers C and D, there was a decrease to 29.41% and 30% (a mean of 29.71%)(Table 4). The decrease in the mean amitraz residue was (32.57- 29.705=2.865) %. This decrease could be accounted for with the extra water taken by the cows. This suggested that a lot of fluid (water) assisted in the elimination of amitraz residue in the cows via urine (mostly) or dung rather than in milk secretion. The mean amitraz for the entire four farmers A, B, C and D) follow the same pattern as shown in Fig.11. Farmer A owned a Jersey cow which had the highest mean amitraz concentration of 0.06 ppm (bf 5.005) compared with the others farmers B, C and D which had 0.0325 ppm (3.95), 0.043115 ppm (4.51) and 0.031667 ppm
(3.52625), respectively. These high values of amitraz concentrations could be accounted for by the high butterfat content over the other cows Ayrshire, Guernsey and Freshian. If milk were to be taken on daily basis, the individual amitraz residue for the farmers A, B, C and D would be the most

toxic, since the values are above the established reference dose (RfD) for amitraz residue, which is at 0.0025 mg/kg/day. From the samples selected, 5 samples were above the MRL, which was 15.625% accounting for the toxification of the milk. Two of the samples had amitraz concentrations of 0.05 ppm, which is the MRL and was 6.25%, a critical point of milk consumption. The remaining 25 samples had amitraz concentration below MR, and which accounted for the safety of milk for consumption. This was about 78.121% of milk samples collected. Amitraz residue was detected in all the samples (M/S), and quantification (ECD) was done in all except 2 samples that is before spraying and the 7th day after spraying for farmer D. Since amitraz residue was below the limit of detection with ECD, it could not have been detected (Table 10 and Figs. 11 and 12).

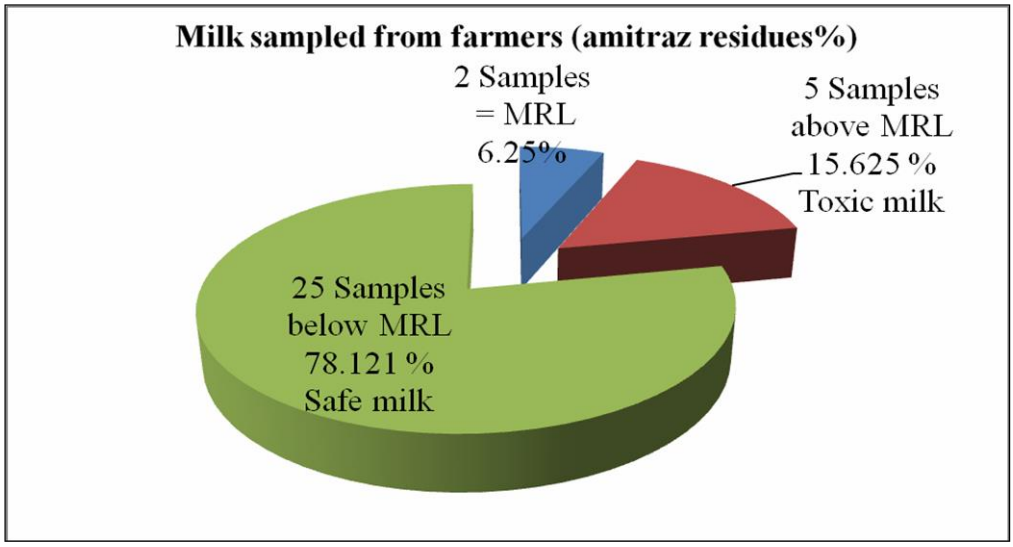


Fig. 12: Milk sampled from farmers with their amitraz residues

Milk sampled from vendors in Eldoret town and its surroundings

The average concentration of amitraz residue from milk samples ranged from (0.02 - 0.05) ppm. It was found that, out of the 48 milk samples 20 had amitraz residue below MRL. This accounted for the 39.58% of the milk samples. Five samples had amitraz residue above MRL, which was 10.42% of samples. Four of the samples had amitraz residue at 0.05 ppm (MRL) a critical point of milk consumption and this was 8.33% (Fig. 13). Amitraz residue was detected but not quantified in 5 milk samples, which was about 10.417% of the milk samples. This was as a result of amitraz residue being below the limit of detection by ECD. Finally there was no detection of amitraz residue in 15 samples, probably because the farmers could have used other types of pesticides or not used them at all. This accounted for the 31.25% of the total

milk samples collected (Figures 13 and 14). In general 58.33% of the samples indicated amitraz residue was detected and quantified, representing 10.42% of the values above the MRL, while the rest were below MRL (Figure. 14). In total 68.75% of milk samples showed some detection of amitraz residue, indicating that amitraz is still the choice of the farmers, (Fig. 15). However, 89.58% of milk samples were within the limits set by FAO, WHO and EPA (MRL = 0.05 ppm). Hence these samples of milk were safe for consumption and only 10.42% of the samples had amitraz residue above MRL. This accounted for the unsafe milk for consumption (Fig. 16). This can cause reproductive, developmental and neurological toxicity risks to the general population (EPA, 1996). It is expected that the percentage of unsafe milk for consumption should be higher than this, due to the low level of butterfat content in the milk sampled, suggesting that a lot of milk is highly diluted with water, thus lowering the amitraz residue in the milk. This gives milk the poor quality, which made it deviate from the normal composition as a unique and ideal food (Table 6).

Fig. 13: Milk sampled from milk vendors with their amitraz residues

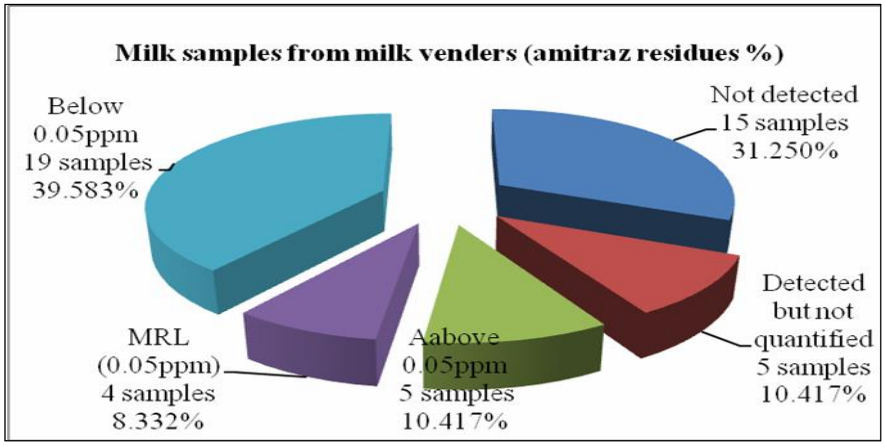


Fig. 14: Distribution pattern of amitraz residue in milk samples

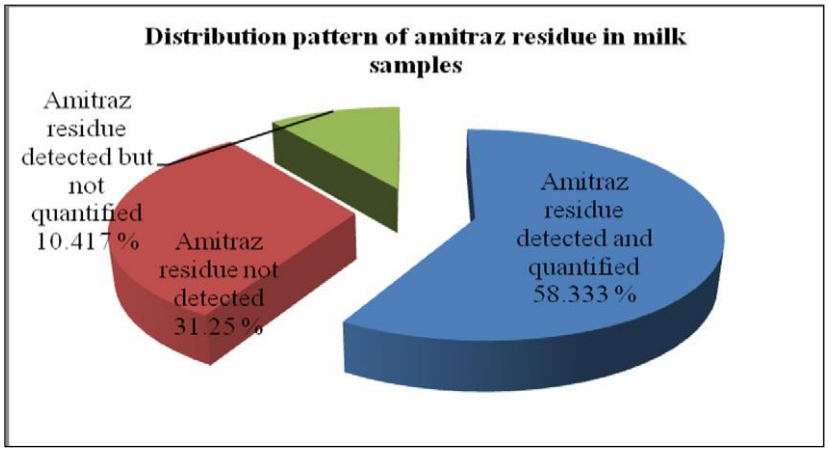


Fig. 15: Use patterns of amitraz based pesticides

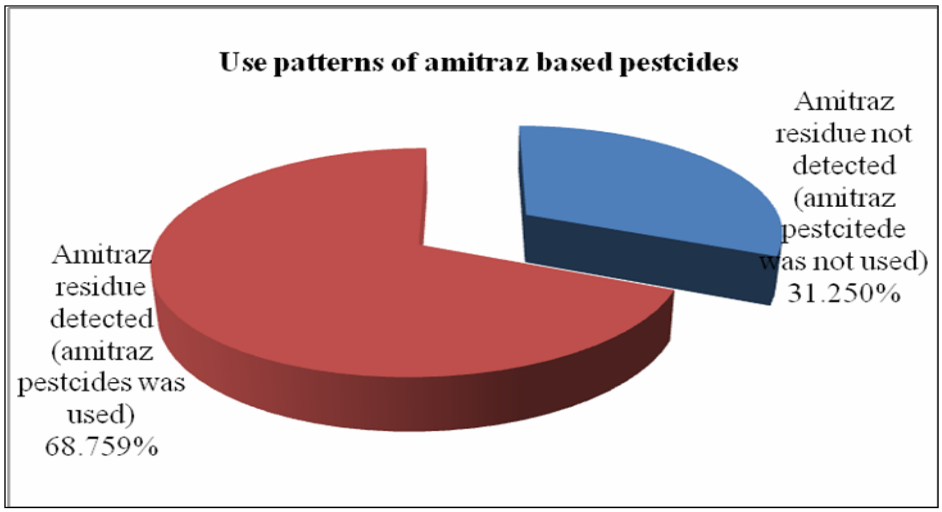
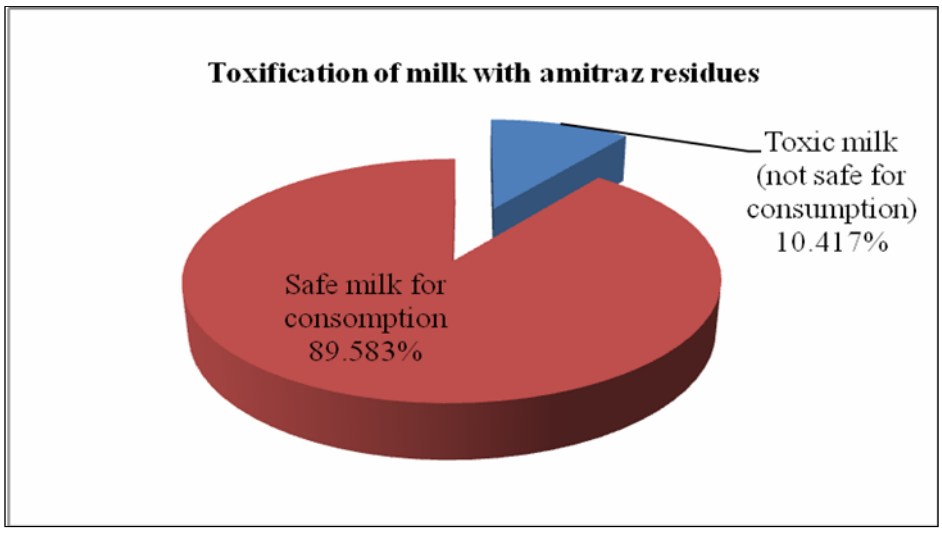


Fig. 16: Toxification of milk with amitraz residues



Sampling areas for milk vendors,Uasin Gishu	**Amitraz concentration and butterfat content**			**Mean**
Beta farm	a c - b f 3.50	0.02 2.50	0.04 3.80	0.0200 3.2667
Chep	a c -	-	-	-

	b f 4.80	5.20	4.50	4.8333
Elgon view	a c 0.22 b f 4.50	0.04 3.58	0.05 4.00	0.0967 4.0267
Hawaii	a c 0.04 b f 2.90	+ 1.90	0.03 2.00	0.0233 2.2667
Huruma	a c 0.10 b f 4.30	0.04 2.55	0.05 3.77	0.0633 3.5400
Junction (Iten/chep)	a c - b f 3.50	- 4.20	- 3.00	- 3.5667
Kamukunji	a c 0.02 b f 2.25	- 2.10	+ 1.90	0.0067 2.0833
Kapsowar	a c 0.04 b f 3.65	+ 3.25	- 3.50	0.0133 3.4667
Kidiwa	a c 0.03 b f 2.56	- 1.90	0.03 2.11	0.0200 2.1900
Langas	a c 0.04 b f 3.50	0.05 3.66	0.03 2.88	0.0400 3.3467
Mail nne	a c - b f 3.56	0.03 3.40	+ 3.88	0.0100 3.6133
Munyaka	a c - b f 2.50	- 2.77	- 2.31	- 2.5267
Road block	a c 0.06 b f 4.50	+ 3.20	0.03 3.45	0.0300 3.7167
Town centre	a c 0.05	0.06	-	0.0367

	b f 4.53	5.17	4.32	4.6733
West	a c 0.04 b f 3.82	0.03 3.50	0.04 3.60	0.0367 3.6400
West Indies	a c 0.07 b f 4.35	0.02 4.54	0.03 4.25	0.0400 4.3800
Overall mean Amitraz concentration and butterfat content				a c 0.0273 b f 3.4398

Note: ac means amitraz concentration.

bf means butter fat.

+ means amitraz was detected but not quantified

- means amitraz was not detected

Relationship between fat content and amitraz concentration

TABLE 7: Milk samples analysed 1 day before and 7 days after spraying

Selected farmer A, B, C and D.							
Before spray	**After spraying**						
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**
ac / bf	**ac / bf**	**ac / bf**	**ac / bf**	**ac / bf**	**ac / bf**	**ac / bf**	**ac / bf**
0.02 /4.97	0.22 /5.00	0.07 /4.93	0.05 /5.15	0.04 /5.10	0.03 /4.99	0.03 /4.96	0.02 /4.94
0.01 /3.93	0.12 /3.90	0.04 /3.95	0.03 /4.15	0.02 /3.96	0.02 /3.94	0.01 /3.90	0.01 /3.87

0.01 /4.40	0.17 /4.50	0.05 /4.55	0.04 /4.52	0.03 /4.48	0.02 /4.53	0.02 /4.60	0.01 /4.50
+ / 3.48	0.10 /3.50	0.03 /3.58	0.02 /3.65	0.02 /3.55	0.01 /3.50	0.01 /3.45	+ /3.50
Correlation between butterfat content and amitraz							
0.89241	0.99294	0.97165	0.99505	0.96754	0.92973	0.94083	0.96892
Mean correlation between butterfat content and amitraz							
0.957383938							
Mean correlation between butterfat content and amitraz from selected farmers							

Farmer A.	**Farmer B.**	**Farmer C.**	**Farmer D.**
0.024458	-0.067311	0.0374	-0.11387

Note: ac means amitraz concentration.

bf means butter fat.

+ means amitraz was detected but not quantified

Correlation between butterfat content and amitraz concentration in milk was determined. showed that butter fat content and other conditions had some significant difference in amitraz concentration. All the butter fat content was found to fall in the range of (2.00 - 5.17) with most milk having butter fat content falling in the range of (2.50 - 3.50). Average correlation between butter fat content and amitraz concentration was found to be 0.957. This shows that an increase in butter fat increases amitraz residue by 95%, hence increases in milk

toxification. Before spraying, the correlation was found to be 0.892 but after spraying from days 1,2,3,4,5,6 and 7, it was; 0.993, 0.972, 0.995, 0.968, 0.941 and 0.969, respectively. The mean correlations between butterfat content and amitraz from selected farmers for the 8 days were, however too low and these were Farmers; A (0.024458), B (-0.067311), C (0.0374) and D (-0.11387). This could be due to greater variation of amitraz concentration with time length that accounts for low correlation values (see Table 7).

TABLE 8: Correlation between butterfat & amitraz residue in milk samples.

Milk sampled from milk vendors	
Percentage of butterfat	**Amitraz concentration (ppm)**
4.50	0.22
2.90	0.04
4.30	0.10
2.25	0.02
3.65	0.04
2.56	0.03
3.50	0.04
4.50	0.06
4.53	0.05
3.82	0.04
4.35	0.07
2.50	0.02
3.58	0.04
2.55	0.04
3.66	0.05
3.40	0.03
5.17	0.06
3.50	0.03

4.54	0.02
3.80	0.04
4.00	0.05
2.00	0.03
3.77	0.05
2.11	0.03
2.88	0.03
3.45	0.03
3.60	0.04
4.25	0.03
Correlation of butterfat and amitraz concentration	
0.445061842	

Milk sampled from milk vendors had low values of correlation (0.445). This could be due to some water being added to the milk which dilutes it and lowers of the butter fat content due to greater variation (Table 8).

A comparison of mean amitraz residue concentration between farms had t_{ext} value of :

Farmers A &B was 19.012

Farmers A &C was 7.083

Farmers A &D was 18.418

Farmers B &C was 13.514

Farmers B &D was 10.890

Farmers C&D was 15.911

Since $t_{ext} > t_{8\ (critical),}$ where $t_{8\ (critical)} = 2.306$ implies that different conditions in the farms, such as rate and frequency of spraying or dipping, feeding, watering of the livestock, cleaning of

udder before milking, time of milking after spraying and the type of cow caused significant difference in amitraz concentration at 5% significant level(Table 9).

TABLE 9: Comparison of mean amitraz concentration between farms.

	Farmer A Jersey cow	**Farmer B Ayrshire cow**	**Farmer C Guernsey cow**	**Farmer D Freshian cow**
Farmer A Jersey cow		19.012	7.083	18.418
Farmer B Ayrshire cow	19.012		13.514	10.890
Farmer C Guernsey cow	7.083	13.514		15.911
Farmer D Freshian cow	18.418	10.890	15.911	

Conclusion

It was found out that about 68.75 % of the milk sampled contained amitraz residue. Before spraying, amitraz was low. There was a sharp increase a day after spraying, after day 2 there was a sharp decrease and the same trend was observed for subsequent days. There was a gradual decrease in concentration of amitraz as the number of days increased, until day 7 when it was the lowest. The average concentration of amitraz residue from milk samples ranged from 0.02 - 0.05 ppm. It was observed that 39.58% had an average of amitraz residue concentration below the WHO, FAO and EPA MRL. However, about 8.33% of samples analysed had an average amitraz concentration of 0.05 ppm, which is the MRL. About 10.42% of samples analysed had an average amitraz concentration in the range of (0.06 - 0.22) ppm, which is above the MRL. These results show that part of the milk sampled did not exceed the WHO, FAO and EPA standards for the amitraz MRL. The implication of this is that there are toxicity effects on consumers of such milk. This high amitraz concentration (0.06- 0.22) ppm could be attributed to the incorrect usage of amitraz based pesticides. The correct recommended rate of usage by manufactures is 2.5 ml/L for dipping and 2.0 ml/L for spraying, at intervals of 2 weeks. The optimum condition taken were from the results for the mean peak areas of derivative formed from reflux times (2 hrs), water bath periods (60 minutes) and water bath temperatures (50 ^{o}C). These had the largest mean peak areas signifying the condition at which maximum amount of derivative

(NH2, 4 X) was formed. The effectiveness of GC/MS and GC/ECD reveal the practicality of analytical method used to determine amitraz residue in milk above MRL. Qualitative analysis done with GC/MS offered an ideal identification of ions or fragments. Quantitative analysis done with GC/ECD has a high efficiency of separation while ECD has a lower detection limit of 0.016 ppm. Splitless mode produced data that was reproducible, as its relative standard deviation was lower than 10%. Percentage recovery was found to be above 80 % the accepted values. This showed the effectiveness of extraction and derivation of amitraz residue in milk. The butter fat content was found to fall in the range of 2.00 - 5.17 with most milk having butter fat content falling in the range of 2.50 - 3.50. A mean correlation between butter fat content and amitraz concentration was found to be 0.957383938 (positive). To minimise milk toxicity, milk should have a withholding period of one day after spraying. Livestock should also be given plenty of water so as to direct most of the amitraz residues through urine rather than in milk secretion.

Acknowledgements

Very special thanks go to Moi University for grant and to carry out this research.

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