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RESEARCH ARTICLE

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Correlation between Chlorpyrifos Residues and Calcium Levels in Milk from Dairy Farms in Nakuru County, Kenya

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Abstract

The use of organophosphate (OP) pesticides in dairy farming has increased due to their availability in the market and low prices. Chlorpyrifos (0, 0 – diethyl 0 - (3, 5, 6 – trichloro – 2 - pyridyl phosphonothioate; CP) is a medium-risk acaricide for humans whose widespread use in agriculture creates various health concerns. In dairy farms, extensive use of CP causes milk contamination and alteration of the composition and chemistry of dairy products. Few studies have examined the impact of CP residues on calcium levels in milk. Therefore, the present study aimed to determine the effect of Chlorpyrifos organophosphate on calcium levels in milk. Fresh milk samples were obtained from 15 Nakuru County, Kenya, dairy farms. HPLC was used to detect and measure CP levels in the milk samples, while calcium concentration was assessed through UV-VIS Spectrophotometry. The results show that approximately 53% of raw milk samples had significant detectable chlorpyrifos levels. All the positive milk samples exceeded the maximum residue limit (MRL). Calcium concentrations in the sampled milk from Nakuru County were not significantly higher or lower than the reference levels. A weak and negative correlation was found between calcium concentration and Chlorpyrifos residue levels (r = -0.2). These findings suggest that milk contamination with organophosphates affects their composition. Therefore, there is a need to properly handle milk to avoid contamination by chlorpyrifos and other pesticides used in agriculture. Besides, milk should be appropriately processed to reduce pesticide residues and metabolites in the final products.

Keywords: Calcium, Chlorpyrifos, Milk Contamination, HPLC, Organophosphate, Spectrophotometry

INTRODUCTION

Pesticides are poisonous compounds that kill or slow the development of pests in animals and crops (Martins et al., 2013; Jawaid et al., 2016). So far, the most extensively used pesticides are those classified as organophosphorus (OPs) (Munoz-Quezada et al., 2013), which account for around 38% of all pesticides used globally (Ambreen and Yasmin, 2021). Their attractiveness stems partly from low persistence time and degradability (Sapahin et al., 2014). OPs are synthetic biogenic chemicals having a binding covalent link between carbon and phosphorus that substitutes the usual phosphate ester's carbon-to-oxygen-to-phosphorus bond (Kazemi et al., 2012; Mutavi et al., 2018). One of the most extensively utilized organophosphates in agriculture is chlorpyrifos (CP). The World Health Organization (WHO, 2018) classify it as a moderate-risk compound. The chemical is fat-soluble; thus, it can get to the body through the skin, finding its way to meat and milk products. As a result, organophosphate residue is often present in fresh and processed milk (Avancini et al., 2013). Some of these residues have been linked to severe health conditions in humans and livestock, including neurological, immunological, and fertility disorders (Bundotich & Gichuhi, 2015).

According to the WHO, the estimated recommended daily intake (ADI) of chlorpyrifos for humans is 0 to 0.01 mg/kg bw, with the acute reference dose (ARfD) at 0.1 mg/kg bw (WHO, 2018). Maximum residual limits (MRLs) for pesticides in animal-derived products, such as milk (0.01 mg/liter), have been established to promote trade and safeguard consumers (Shaker and Elsharkawy, 2015). However, several investigations have shown that contaminated dairy milk may have levels over this limit, posing a serious health risk (Bedi et al., 2018). Pesticides were one of the causes of acute poisoning in Nakuru County, according to Bundotich and Gichuhi (2015). Since milk products are extensively consumed in Kenya, CP residues in milk represent a public health problem. Milk and milk products are an essential part of human nutrition worldwide, contributing a large portion of proteins in the diet (Bedi et al., 2015). Therefore, milk must be hygienic without any hazardous chemical or microbial contamination. However, the excessive use of pesticides and acaricides has retained residues in milk and its processed products (Chen et al., 2014). As a result, lactating cows can be exposed to OP in many ways, including application on the animal body, ingestion of feed, fodder, and water, and in animal processing areas (Bedi et al., 2015).

Previous studies have explored the impact of acaricide residues on various milk components, including proteins, carbohydrates, and lipids (Kazemi et al., 2017; Bedi et al., 2018; Raheem & Niamah, 2021). However, no study has examined how the residues and their metabolites affect essential minerals, such as calcium. Much focus has been put on calcium because many adults do not consume the recommended quantity of the nutrient, leading to calcium deficiency that contributes to the development of disorders. Calcium deficiency in the human body is implicated in diseases such as osteoporosis, hypertension, and amyotrophic lateral sclerosis (Pettifor, 2014; Cormick and Belizán, 2019). Cow milk is the most important source of calcium for human consumption (Pšenková et al., 2020). Few studies in Kenya have examined the levels of chlorpyrifos in milk and milk products and their effects on milk composition and chemistry. Therefore, the current study aimed to assess presence of CP residues in milk from farms within Nakuru County through HPLC analysis and correlate them with calcium concentration, as measured through UV-VIS Spectrophotometry.

MATERIALS AND METHODS

Study Area

The empirical study was conducted in Nakuru County, Kenya (Figure 1). The study site was chosen because Nakuru County is one of the leading dairy farming regions in the country.

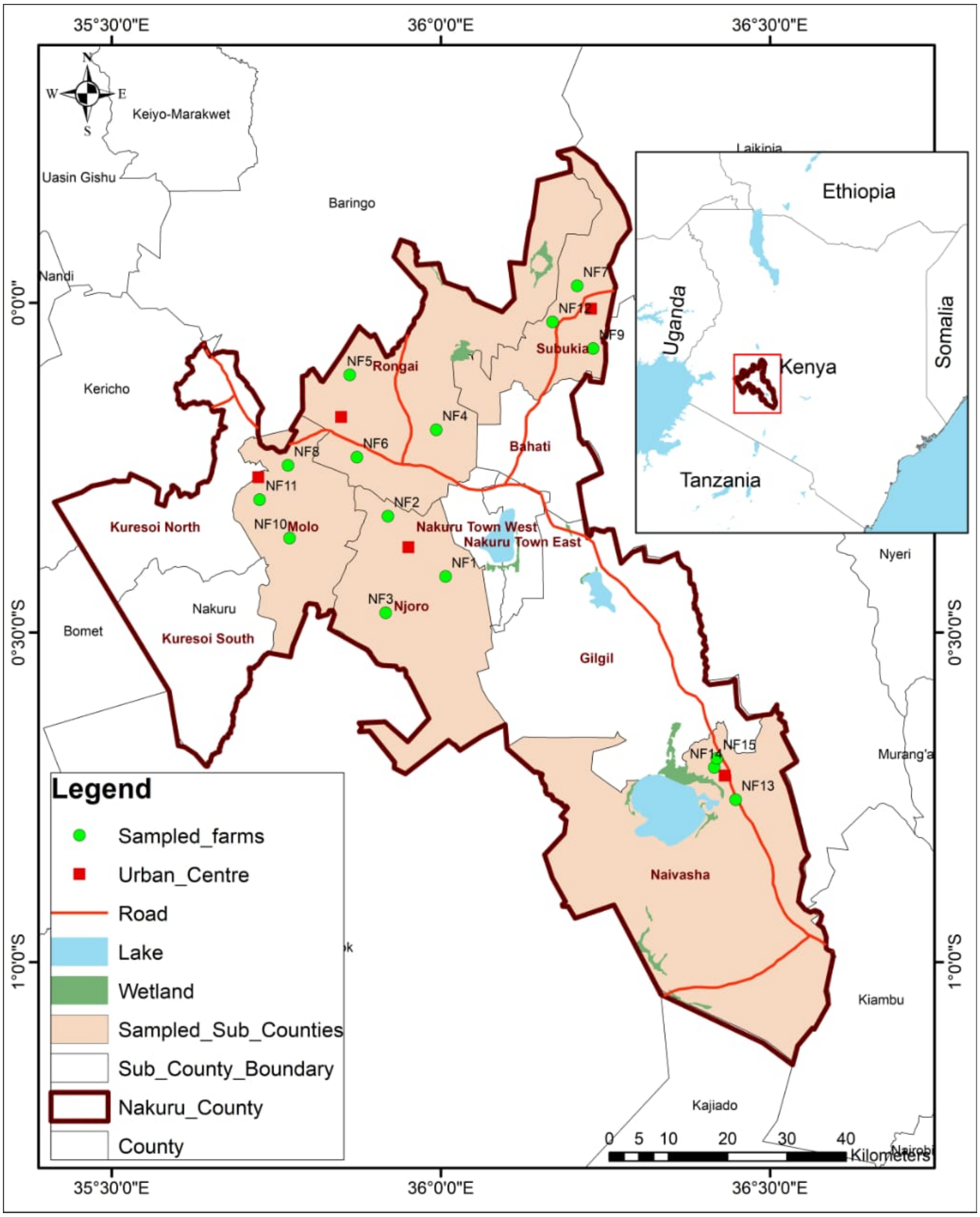


Figure 1: **The study area showing the sampling sites in Nakuru County, Kenya.**

Sample Collection
Nakuru County was purposely selected because it was considered one of the leading producers of dairy products, with intensive livestock farming and extensive use of agrochemicals. A random sampling technique was used to select a sample of large-scale dairy farmers in the county. The study was carried out using a cross-sectional design. Fifteen dairy farms in Nakuru County were chosen because they had a recent or current CP acaricide use history. Milk samples were collected via stratified random sampling from five sub-counties of Nakuru County: Molo, Njoro, Rongai, Subukia, and Naivasha, based on stratified sampling, in which three farms were selected from each sub-county. The sampling size was informed

by a pre-study survey that estimated the number of dairy farmers using CP regularly to be about 150. Thus, fifteen farms (10% of the target population) were purposively considered a representative sample. The chosen farms had used OP acaricides in the previous year. Fresh milk samples, 100 ml from each dairy farm, were collected aseptically in duplicates directly from the farms immediately after milking, and they were kept in sterilized screw cap plastic bottles. The collected samples were coded for identification and stored in a cool box with ice packs during fieldwork. After that, they were transported to the Research laboratory at Mount Kenya University, Thika, where they were stored in a freezer (-20°C) until analysis.

Chemicals and Reagents
Analytical grade Chlorpyrifos standard (99.5% purity) used in the analysis was sourced from Sigma-Aldrich Corporation. Commercial grade Chlorpyrifos (Duodip 55% CP) was obtained from a retail outlet in Thika. The Calcium Kit used to determine calcium levels was from Precise Labs, Kenya. All reagents used in the study were prepared using the manufacturers' instructions. Water and acetonitrile used in the analyses were HPLC grade.

Determination of Calcium Levels in Milk Samples
Calcium levels were determined using the UV-VIS Spectrophotometer (AVI 2700 double beam, Thermo Fisher Scientific). The Eurochem Calcium Kit was used for the quantification. The principle of the kit is that calcium in an alkaline medium combines with O-Cresophthalein Complexone to form a purple-colored complex (Calcium + OCPC = Purple colored complex) (Gitelman, 1967). Therefore, the quantity of calcium in the sample is directly proportional to the intensity of the color generated. First, one ml of milk was diluted with nine ml of distilled water, and one ml of the diluted milk was centrifuged for five minutes at 2000 rpm. Then, the obtained supernatant was transferred to clean test tubes. After the mixing, incubation was done for 5 minutes at room temperature. Afterward, absorbance readings were done within 60 seconds, with the test sample and standard compared with the blank. The following formula was used to determine the calcium concentration in the sample.

$$Calcium\ Concentration = \frac{Absorbance\ of\ the\ sample\ X\ 10\frac{mg}{dl}X\ 10}{Absorbance\ of\ the\ standard}$$

Determination of Chlorpyrifos Levels in Milk
Sample Extraction
An optimized extraction method described by Fagnani et al. (2011) was used for all samples described herein. Samples were first defrosted, and 1ml of each sample was pipetted into a screw-top centrifuge tube. Afterward, 5 ml of a solution of acetonitrile and phosphate buffer (90%:10%, pH 4.5) was added. The mixture was gently swirled, sonicated for 10 minutes, and vortexed for 15 minutes at 1200 rpm. After that, centrifugation was done at 2000 rpm for 5 minutes. The supernatant was transferred into a screw cap test tube. Double extraction was done by adding 5 ml of the premixed solution of acetonitrile and phosphate buffer to the original sample, and the procedure was repeated (Hamid et al., 2021). The supernatant was added to the test tube. An aliquot of the extract was filtered through a 0.45 µl Teflon syringe filter into an HPLC vial (Harshit et al., 2017).

HPLC Analysis
High-quality liquid chromatography was utilized to identify and quantify CP in milk samples (Mauldin et al., 2006). A reverse-phase C-18 column was utilized in a Shimadzu HPLC with two LC-10AT VP pumps and a variable wavelength UV detector. The Lab Solutions software was installed on the HPLC equipment (Shimadzu). At a flow rate of 1ml/min, the mobile phase components (75 percent acetonitrile and 25% PO_4 buffer) were injected into the column. The temperature was kept at 30 degrees Celsius. The UV was maintained at 230 nm, and 20 µl of samples

were sequentially injected and analyzed by reverse-phase HPLC. Retention times and peak areas were used to identify and quantify compounds compared with the reference standards (Harshit et al., 2017).

The chlorpyrifos standard was made by dissolving it in acetonitrile and serially diluting it to generate 10 ppm solutions, which were then put into an HPLC vial (Chen et al., 2014). Peak area estimates after five injections of the standard solutions to create two ppm, four ppm, six ppm, eight ppm, and ten ppm were used to assess the calibration curve's linearity. Analyses were carried out in batches, with a method file kept in the machine software as a template. The peak area was computed for each sample, and the calibration curve for concentration vs. response area/peak area was shown. The standard deviation was utilized to determine the precision of the analyte, and an external standardization technique with a standard deviation of less than 0.05 was employed. Calibration curves were developed independently for each analysis day to accommodate for daily differences.

Statistical Analysis

The data obtained in the study were analyzed for descriptive and inferential statistics. Descriptive statistics included mean and standard deviation, while inferential statistics was by Pearson Correlation, which was used to show the relationship between Calcium and CP. All statistical analyses were carried out using the Statistical Package for Social Sciences (SPSS) version 24.

RESULTS

Determination of Calcium Levels in Milk

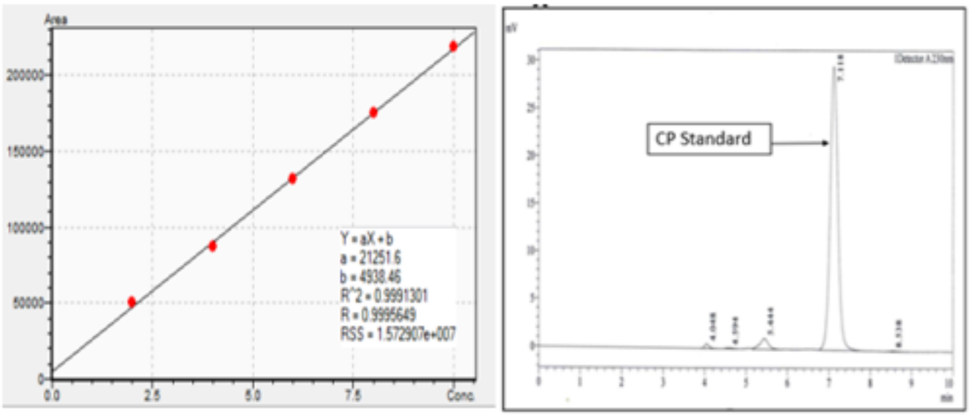
Each of the 15 milk samples was analyzed in duplicates using the UV-VIS Spectrophotometer, and the mean was used to determine the calcium concentration, as shown in Table 1. Most milk samples had calcium concentrations within the normal range of 100-250 mg/dl. The mean calcium concentration of the samples was 275.87 mg/dl, which is not significantly different from the reference value of 120 mg/dl. However, there were two samples with extremely high calcium concentrations (NF5 and NF13) and one with deficient levels (NF9).

Table 1: Calcium Concentration in milk samples from Nakuru County

Sample Code (n = 15)	Mean Calcium Concentration (mg/dl)
NF	198.28 ± 7.02
NF2	264.66 ± 14.20
NF3	295.16 ± 12.00
NF4	231.90 ± 13.43
NF5	544.54 ± 12.02
NF6	197.97 ± 6.86
NF7	346.17 ± 4.93
NF8	231.66 ± 8.82
NF9	85.72 ± 7.11
NF10	124.80 ± 7.21
NF11	192.12 ± 12.70
NF12	262.24 ± 7.67
NF13	603.74 ± 5.62
NF14	215.99 ± 8.00
NF15	343.06 ± 5.65
Mean	275.87
P value	>0.05

Calibration Curve and Retention time of Chlorpyrifos Standard

A standard calibration curve (Figure 2) was obtained to quantify the samples' CP residues by running different standard dilutions. Chlorpyrifos was detected at the 7th minute, with the largest area under the curve.



A B

Figure 2: A- Calibration Curve of Chlorpyrifos Standard and B- Chromatogram with a retention time of 7.1 minutes.

Presence of Chlorpyrifos Residues in Milk Samples

Chlorpyrifos residues were found in raw milk samples from eight (8) out of the 15 farms, representing 53.3% of the samples (Table 2). Residues were detected in raw milk samples from all the five sub-counties: two farms each for Njoro, Rongai, and Naivasha, and one farm each for Molo and Subukia. Thus, it was determined that there is no significant difference in CP contamination across the sub-counties in Nakuru County ($p>0.05$). A t-test showed that the mean CP residues in milk from Nakuru county were significantly higher than the maximum residue limits of 0.01mg/liter ($p<0.01$). A Chromatogram of a positive milk sample is shown in Figure 3.

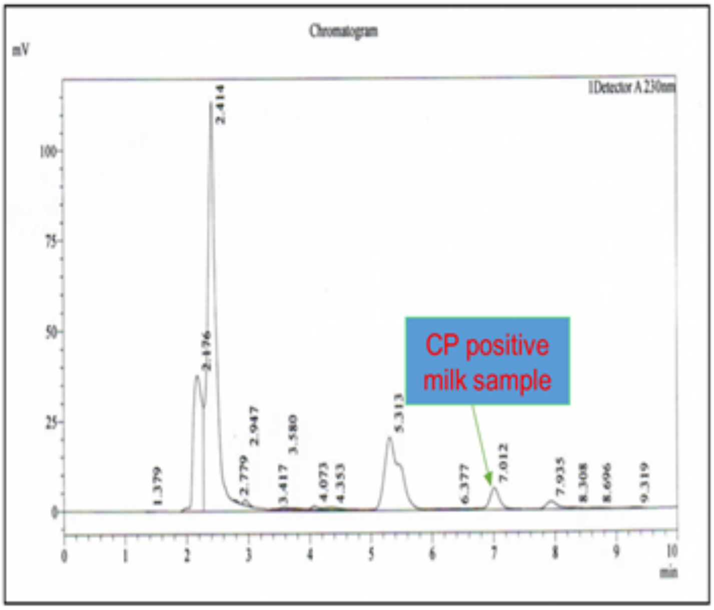


Figure 3: Chromatogram of a positive milk sample.

Table 2: **Chlorpyrifos Residue Levels in Raw fresh milk-based on HLPL analysis**

Farm Code	Sub-county	Mean CP in samples (mg/l) and SD (n = 3)
NF1	Njoro	2.623 ± 0.738
NF2	Njoro	2.721 ± 0.702
NF3	Njoro	BDL
NF4	Rongai	2.163 ± 1.031
NF5	Rongai	BDL
NF6	Rongai	0.9275 ± 1.30
NF7	Subukia	1.518 ± 1.031
NF8	Molo	BDL
NF9	Subukia	BDL
NF10	Molo	BDL
NF11	Molo	2.707 ± 0.351
NF12	Subukia	BDL
NF13	Naivasha	1.194 ±1.052
NF14	Naivasha	1.528 ± 0.875
NF15	Naivasha	BDL
% Positivity		**53.3%**
Mean		**1.923**
P values		>0.05

Key: NF means Nakuru Farmer, and BDL means Below Detection Limit

Correlation between Calcium Levels and Chlorpyrifos Residue Levels

To determine if pesticide residues affect the calcium concentration in cow milk, a twotailed Pearson correlation analysis was executed using SPSS 24 at a significance level of 0.05 (Table 3).

Table 3: Correlation of Calcium Levels and Chlorpyrifos Residue Levels

Correlations			
		Chlorpyrifos Levels	**Calcium Concentration**
Chlorpyrifos Levels	Pearson Correlation	1	-.199*
	Sig. (2-tailed)		.636
	Sum of Squares and Cross-products	3.642	-64.689
	Covariance	.520	-9.241
	N	8	8
Calcium Concentration	Pearson Correlation	-.199*	1
	Sig. (2-tailed)	.636	
	Sum of Squares and Cross-products	-64.689	28894.585
	Covariance	-9.241	4127.798
	N	8	8

As per the results, the correlation coefficient (r) was -0.199, indicating that the two variables, Chlorpyrifos level, and Calcium concentration, have a fragile negative relationship. Secondly, it was reviewed whether pesticide residues (Chlorpyrifos) significantly affect the concentration of calcium in milk. As per the results, the p-value was 0.636, more significant than 0.05. Therefore, it was affirmed that pesticide residues (Chlorpyrifos) in milk's calcium concentration are insignificant. The Pearson Correlation Coefficient scatter plot is shown in Figure 4.

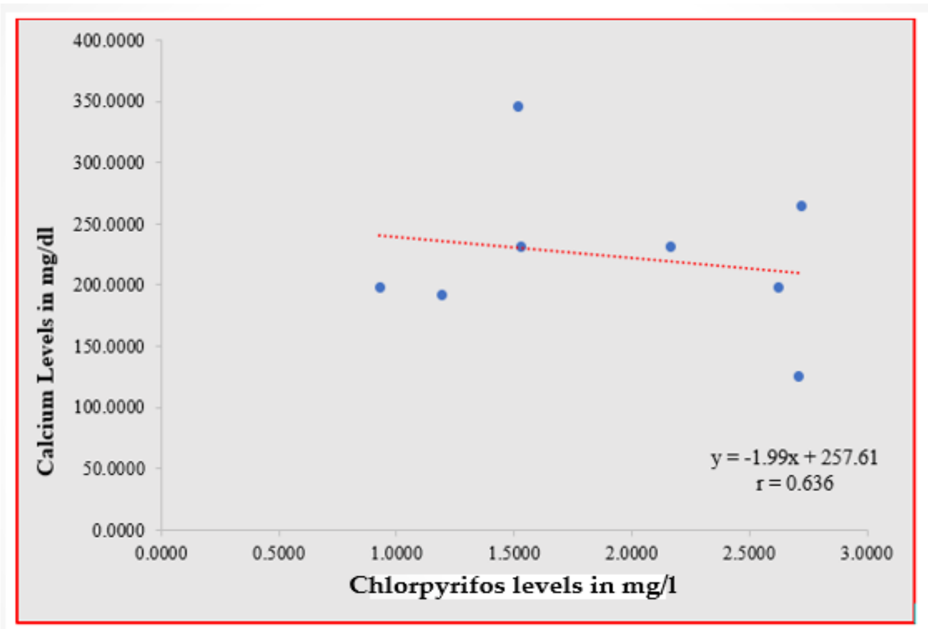


Figure 4: Pearson Correlation Coefficient scatter plot showing line of best fit for correlation between chlorpyrifos and calcium.

DISCUSSION

To our knowledge, this is the first research to investigate the impact of chlorpyrifos on calcium levels in cow milk. The results of this investigation demonstrate that raw milk from Nakuru County has large quantities of chlorpyrifos residues, making it unfit for human consumption. The results corroborate the research by Abdel-Wareth & Abd El-Hamid (2016). They found that only a tiny fraction of pesticides sprayed effectively reach their target, with the bulk distributed in the environment. Chlorpyrifos levels were more significant than the WHO recommended by the MRL (0.01 mg/liter). The results align with previous research, showing widespread pesticide contamination in dairy milk (Aslam et al., 2013; Sahin et al., 2017). In addition, the current study's results are consistent with earlier studies that have shown that the concentration of OP residues in milk from farms where pesticides are used exceeds the maximum residue limits (0.01 mg/liter) (Aslam et al., 2013; Deti et al., 2014; Bedi et al., 2015; Jayasinghe, 2019). In a local context, the results support those of Miriti et al. (2014) and Anode et al. (2018), who found considerable quantities of organochlorides and organophosphates in Nakuru County soil samples.

Residues of chlorpyrifos were identified in raw milk samples from eight farms, accounting for 53.3 percent of the total. The study's findings corroborate a pre-study survey that revealed that most pesticides used in Nakuru County are from the OP class, particularly chlorpyrifos. The amounts of CP found in this research were more significant than those found in fresh milk by Bushra et al. (2014), which ranged from 26 to 45 ng/gm, and Jayasinghe et al. (2019), which averaged 0.0264. In studies that have compared raw milk and value-added products, residues are often higher in the former because high-value product development procedures, such as pasteurization and homogenization, result in the removal of OP residues (Bajwa & Sandhu, 2011). Furthermore, OP acaricides have a short half-life in the environment, which explains why they are found in raw milk but not in preserved long-life milk products (Jayasinghe, 2019).

In the typical blood plasma of cows, the Ca concentration is tightly regulated between 8.5 to 10 mg/dl (2.1 to 2.5 mM) (Wynn et al., 2015). In milk, average levels are between 96 and 260 mg/dl (Gaucheron, 2005). In the present study, the average calcium levels were 279.27 mg/dl, slightly above average. Correlating with CP residue levels, it was noted that the presence of chlorpyrifos was negatively correlated with Calcium levels, although the relationship was weak and insignificant. Previous studies have shown that the composition of milk, including calcium levels, can be affected by various factors, including the type of food, pre-partum nutrition, the environment, and breed (McGrath et al., 2015; Linn, 2016; Albani et al., 2019). According to Linn (2016), diets that reduce milk's fat composition also lower soluble calcium in milk. Zwierzchowski & Ametaj (2019) reported that agricultural and urban environmental pollution could also affect milk composition. Hidiroglou & Proulx (1982) listed calcium, magnesium, and phosphorus as some of the elements affected by pesticide contamination.

Although the present study found only a weak correlation, it is possible that the findings were affected by the petite sample size. Besides, two samples had relatively higher levels of calcium (NF5 = 544.54 mg/dl and NF13 = 603.74 mg/dl). These values were significantly higher than the reference value of 120 mg/dl. Various factors are responsible for this disparity, including breed and genetics, management, environment, the health of the cows, and nutrition (Staufenbiel & Karl, 2017). As noted by Linn (2016), the type of foliage cows feed on can affect milk composition, including the levels of various minerals. Future studies should include positive and negative controls to ascertain a causal relationship between pesticide residues and calcium in milk.

CONCLUSION

The present study explored chlorpyrifos pesticide residues as environmental pollutants because their residues persist in the environment for a long time, ending up in animal feeds. These chemicals and their metabolites accumulate in the bodies of animals, where they pass into milk and milk products. There are significant Chlorpyrifos residues and their metabolites in raw milk in Nakuru County. A correlation analysis showed that residues negatively affect calcium levels in the milk. Considering milk's critical role in the human diet and the importance of calcium in the body, the presence of these residues and metabolites is a severe concern for public health and nutrition. Therefore, our findings have implications for public policy, especially concerning the prevention of milk contamination, regulation of pesticide usage, and proper testing of milk and milk products for contaminants to safeguard public health. Considering the present study's findings only provide preliminary insights, there is a need for further studies to explore the phenomenon in detail. For instance, large-scale, cross-sectional studies are needed to determine organophosphate contamination in milk in the entire country. Also, there is a need to explore how pesticide contaminants in milk and milk products affect other essential minerals in milk, including potassium and magnesium.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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