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DETERMINATION OF ANTIBIOTIC RESIDUES IN COW MILK BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHOD

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ABSTRACT

Antibiotics are used as growth boosters, illness prevention and treatment in animal agriculture. The majority of cattle farmers take advantage of the system and do not adhere to the withdrawal periods. These antibiotics are identified as residues in ready-to-eat meat, causing negative consequences in human beings. The majority of the study on determining antibiotic residues in ready-to-eat meat relied on thorough research and paid little attention to animal products such as cow milk. The goal of this study was to use a method that could be used on a regular basis to determine the amounts of antibiotic residues in cow milk marketed to the general population in Ibadan. These antibiotics residues (chloramphenicol and erythromycin) were extracted from cow milk by centrifugation and liquid-liquid extraction using acetonitrile. Vacuum-liquid chromatography was used to clean the extracts, and 10mL of a 1:1:1 v/v mixture of n-hexane, chloroform, and methanol was used. After that, a high-resolution liquid chromatography analysis was performed on the extracts. All of the samples included residues of chloramphenicol (CAP), with mean values ranging from 91.4610.89 to 692.6616.74 ng/g, while erythromycin (ERY) was not observed. All of the samples contained chloramphenicol, proving that cow farmers had been abusing it.

Keywords: Antibiotics, HPLC, Milk, Residues, Vacuum Liquid Chromatography.

INTRODUCTION

Food safety called for major concern in recent years, especially in light of several food crises that have been impacted public health around the world. As a result, Scientists and Regulatory organizations must detect any potential consumer dangers associated with food consumption. Resistance to antibacterial agents is on the rise, posing a serious threat to human health. As a result, new treatments to combat these issues are in high demand, but as past experience has shown, resistance to new drugs develops quickly.

Antibacterial medications have traditionally been produced based on their capacity to limit bacterial reproduction, and this remains the foundation of most antibacterial drug discovery efforts [8]. Infectious diseases continue to be the cause of morbidity and mortality in humans and animals, particularly in the poor Nations. The fact that many pharmaceutical companies have developed a number of new antimicrobial medications in recent years, microorganism resistance to these drugs has increased [5].

Antibacterial or antimicrobial chemicals can be natural, synthetic or semi-synthetic. The term "antibiotic" sometimes used interchangeably with the more generic term "antibacterial" to refer to the entire class of medications. Antibiotics, such as chloramphenicol for mastitis and erythromycin for intestinal infections, are commonly used in the treatment of bacterial disease. They've also been

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employed as growth-promoting feed additives. The misuse of antimicrobials in human medicine has long been blamed for the development of antibiotic resistance in bacteria, but the link between agricultural antimicrobial usage and antibacterial resistance in humans is also a source of worry. As a result, precise detection of low quantities of these antibiotics in food is critical [1]

Antibiotics are commonly used nowadays, and inappropriate use can result in milk contamination at the farm level. Antibiotics are regularly used as antimicrobials for mastitis therapy in dairy cows, and thus they are mostly discovered type residues in cow milk [2]. Residues are a source of concern because of the potential for ill effects on persons who are allergic to antibiotics, as well as antibiotic-resistant bacteria development. In humans, this bacterium can cause aplastic anemia as well as have a carcinogenic effect. These factors lead regulatory bodies like the European Union to establish Maximum Residue Limits (MRLs) and Acceptable Daily Intakes (ADI) for the quantity antibiotic agents in milk [4].

The use of antibiotics in medicine is crucial, yet bacterial resistance to them is a real threat. Bacteria that are resistant to routinely used medications are known as antibiotic-resistant bacteria. Repeated exposure to the same antibiotics can cause bacteria to adapt and stop responding to the medication. There are several mechanisms for bacteria to develop antibiotic resistance. For instance, they have an internal mechanism to alter their structure so the antibiotic is no longer effective, or they create strategies to neutralize or inactivate the antibiotic. Furthermore, bacteria can exchange genes that code for antibiotic resistance, allowing bacteria that have never been exposed to an antibiotic to pick up resistance from those that have. When antibiotics are used to treat diseases where they are ineffective, the problem of antibiotic resistance gets exacerbated [11]

The goal of this study is to determine the concentrations of residual chloramphenicol and erythromycin in the cow milk using vacuum liquid chromatography clean-up of extracts and subsequent analysis by HPLC

MATERIALS AND METHODS

Chemicals used

Sigma-Aldrich provided the high purity antibiotic standards (98.9%) for Chloramphenicol and Erythromycin. The following chemicals were used; methanol, acetonitrile, deionized water, n-hexane, chloroform, phosphoric acid, potassium phosphate salt, distilled water and ammonium acetate. The solutions were all created by dissolving the necessary weights in distilled water, and all of the compounds were of analytical quality.

Samples Obtained and Preparation

Sixty (60) samples of cow milk were obtained from three separate markets in the Ibadan metropolis. Each sample of cow milk was placed in a fridge with an ice pack and labeled, sterile plastic bottles and then taken to a laboratory for analysis right away. By diluting 15 mL of concentrated

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ammonium solution in 100 mL of distilled water and making up to the required volume in a 1-liter volumetric flask, the mobile phase and reagent solutions, M = 0.2 0.02 M ammonium acetate buffer, were created. In 1 litre volumetric flask, 3.484 g potassium phosphate salt was dissolved in 1000 mL distilled water to make potassium phosphate buffer solution.

High Performance Liquid Chromatography Analysis, Extraction, and Clean-up

The extraction and clean-up procedures for chloramphenicol and erythromycin in milk samples were based on [9] and [6].

5 g of milk samples were carefully weighed into centrifuge tubes, which were then mixed with 20 mL of acetonitrile, homogenized, subjected to vortex for 10 minutes, then centrifuged for 3000 rpm for 10 minutes at 6°C. Rotary Evaporator was used, supernatant top layers were collected and evaporated to dryness. The dried extracts were pre-adsorbed with a small amount of column grade silica gel to generate a slurry, which was then loaded onto a sinta with TLC grade silica gel as a stationary phase (diameter 3.0 cm and length 5.3 cm). The glass column was eluted progressively with increasing polarity solvents, ranging from n-hexane to a 10mL combination of hexane, chloroform, and methanol (1:1:1, v/v). Before injecting 20µL of extracts into the HPLC column, the eluent for each extract was collected and heated to 40°C using a Rotary Evaporator.

System and process for HPLC

On a Cecil-Adept system, HPLC was performed (Ce-4900). A binary pump (Ce-4100), degasser (Ce-4020), C18 Column (Ce-4600: 150x4.6mm), and UV-detector was included in the system (Ce-4300). The Power Stream V4.2 software was used to process the data.

The mobile phases were a 75:25 v/v mixture of acetonitrile and water, and a 65:25:10 v/v mixture of acetonitrile, water, and 0.2 M ammonium acetate.

Mobile phases were sonicated for 30 minutes to remove gas before application. 278nm and 215nm were used as the excitation and emission wavelengths for detection. The injection volume and the flow rate were 20µL and 1 mL/min respectively.

Preparation of standard curves and working solutions.

0.01g of chloramphenicol was dissolved in acetonitrile and water at a 75:25 (v/v) ratio to produce a fresh chloramphenicol standard solution. The concentrations of 50, 25, 12.5, 6.25, and 3.125g/mL, which were acquired through serial dilutions, were used to construct the standard curves. 2.5mg of the reference standard were mixed in a mixture of acetonitrile, water and 0.2 M ammonium acetate to produce a 50g/L erythromycin standard stock solution (65:25:10). Working solutions of 30, 20, 10, and 5g/mL were produced in a 25mL volumetric flask by dilutions of 15, 10, 5, and 2.5 mL with acetonitrile.

Validation of method

Chloramphenicol and erythromycin standard solution concentrations and observed peak areas

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were utilized to create linear (R2=0.996 and 0.997) calibration curves. The calibration curves were used to extrapolate CAP residue levels. The following are equations to establish the Limit of Detection and Limit of Quantification for CAP residues:

$$\text{LOD } (\mu g/mL) = \frac{3 \times S.D}{\text{Slope}}$$

$$\text{LOQ } (\mu g/mL) = \frac{10 \times S.D}{\text{Slope}}$$

Recovery Study

The following equation was used to determine the chloramphenicol recovery method, [10].

$$\text{Vol. of Spiking Soln} = \frac{\text{spike conc. desired} \times \text{vol. of sample to which spike is added}}{\text{Conc. of the spiking solution}}$$

To calculate the percent recovery of the spike as follows:

$$\%R = \frac{(\text{spiked sample result - unspiked sample result}) \times 100}{\text{Known spike added concentration}}$$

RESULTS & DISCUSSION

Nine samples (from Ojoo, Sango, and Bodija) were tested for residual chloramphenicol, and all of them were found to be positive.

As indicated in Table 1, the mean concentration of drug residue in the samples ranged from 91.4610.89 to 692.6616.74 ng/g, with the maximum quantity being 692.66 ng/g, which is higher than the European Union zero maximum residue limits (EU). When the amounts of chloramphenicol residues in the three samples purchased from different markets were compared, it was discovered that samples purchased from Ojoo market area had a higher concentration than samples purchased from Bodija and Sango. These disparities in residue levels reflect changes in animal husbandry across the state. The findings of this study can be compared to those of [3] who evaluated forty samples of milk and milk products using the LC-MS method and found that three samples had CAP values of 0.37, 0.29, and 0.39 g/kg, respectively. The presence of this drug residue (Chloramphenicol) in cow milk can be linked to inappropriate drug use and a lack of withdrawal period following treatment by cattle ranchers before the milk is sold to the general population. This showed that despite being known to be carcinogenic, concerned regulatory organizations like NAFDAC do not ban or regulate it.

Erythromycin residues were not found in any of the samples tested, according to the chromatogram data obtained from the study. Negative samples could indicate that erythromycin residues are present in the cow milk but at the quantities below the detection limit of the HPLC system used in this study.

Accuracy is a measure of how closely an experiment's outcome matches the expected outcome. Replicate analysis containing known levels of the analyte determines accuracy, which is commonly reported in terms of percentage recovery as indicated in Table 2. [7]. Precision, on the other hand, is a measure of this variability that may be broken down into three categories: repeatability, intermediate precision, and reproducibility. It's also possible to express it in terms of the coefficient of variation (CV). Table 1 shows the coefficient of

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variation for chloramphenicol, with values ranging from 2 to 11 percent, demonstrating that the approach is accurate.

Table i: The summary of Chloramphenicol residual quantities in cow milk samples, with concentration ranges, mean concentrations, standard deviations, and coefficients of variation.

Sample	Concentration(ng/g)		CV (%)
	Range	Mean± SD	
C1	675.44-708.88	692.66±16.74	2.42
C2	323.85-357.48	342.44±14.36	4.19
C3	82.59-103.62	91.46±10.89	11.9

Table ii: The Study of Recovery (5 g was used for chloramphenicol percentage recovery)

Analyte	Conc. of Un-spiked (µg/mL)	Amt. Spiked	Conc. of Spiked (µg/mL)	% R
CAP	1.7869	0.1mL, 2µg/mL	3.5216	86.7

Table iii: This demonstrates the calibration curve equation as well as the antibiotics' Limit of Detection (LOD) and Limit of Quantification (LOQ).

Antibiotic	Std calibration equation (y =mx + c)		R^2	LOD (ng/g)	LOQ (ng/g)
	m	c			
CAP	12.67	0.00	0.996	0.399	1.331
ERY	849.30	0.00	0.997		

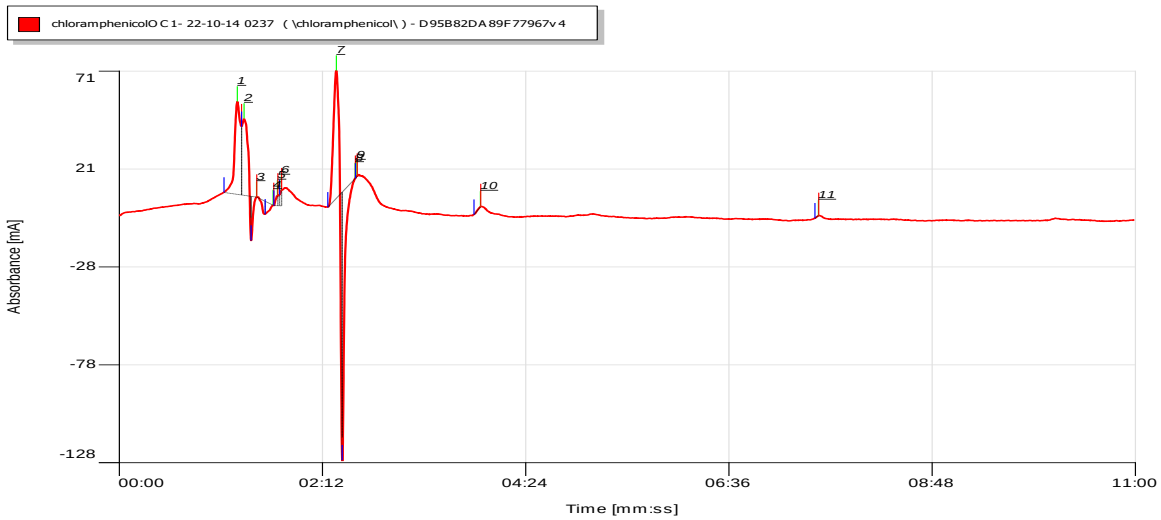

Figure i: Shows the chromatogram report of chloramphenicol from Oojo market

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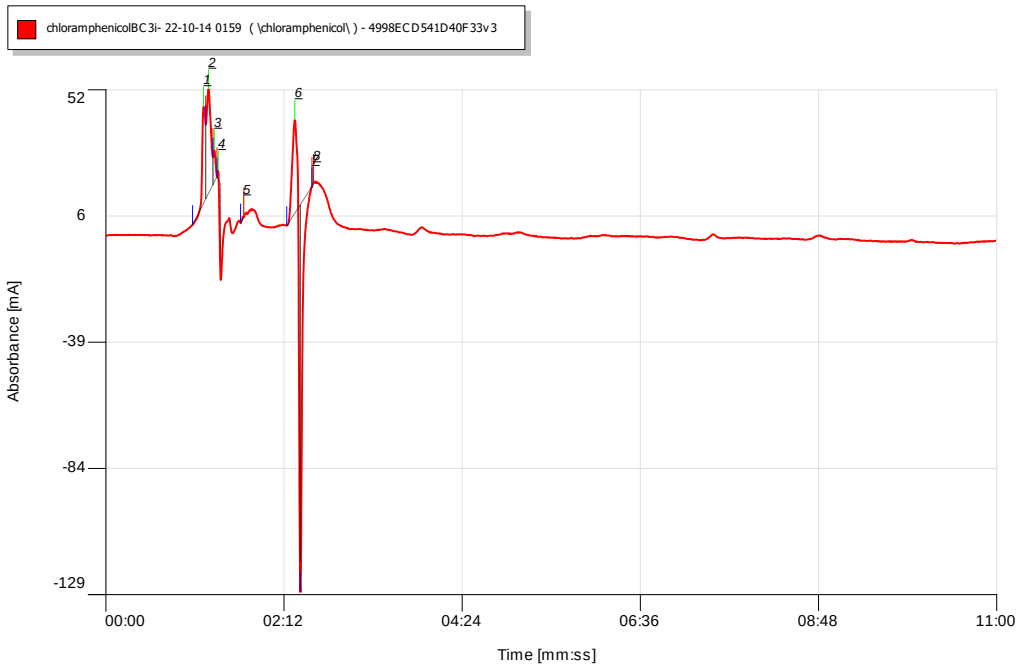

Figure ii: Shows the chromatogram report of chloramphenicol from Bodija market

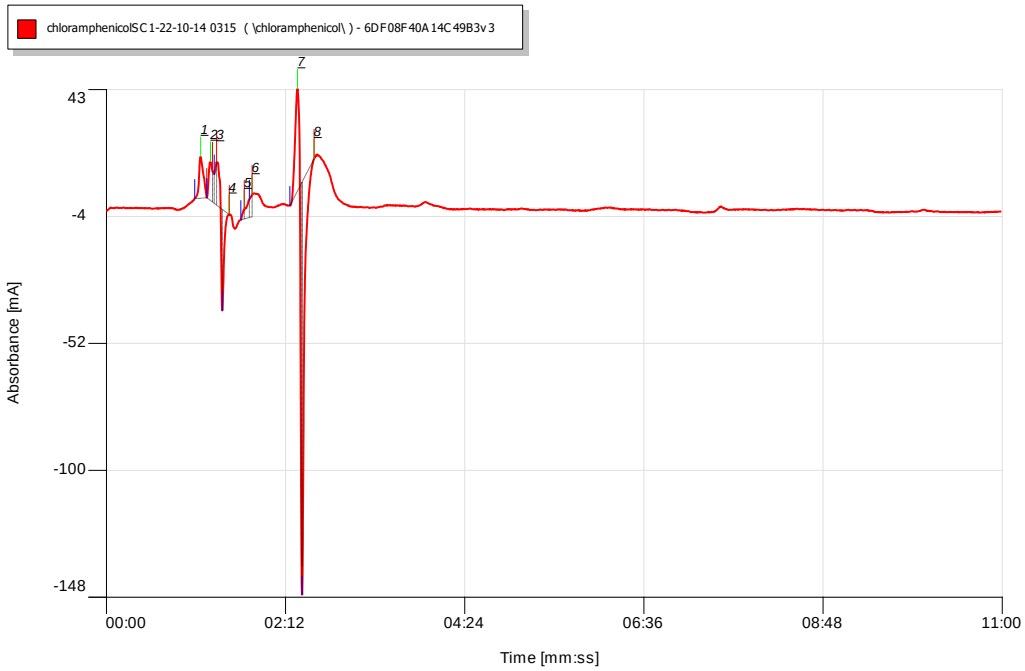

Figure iii: Shows the chromatogram report of chloramphenicol from Sango market

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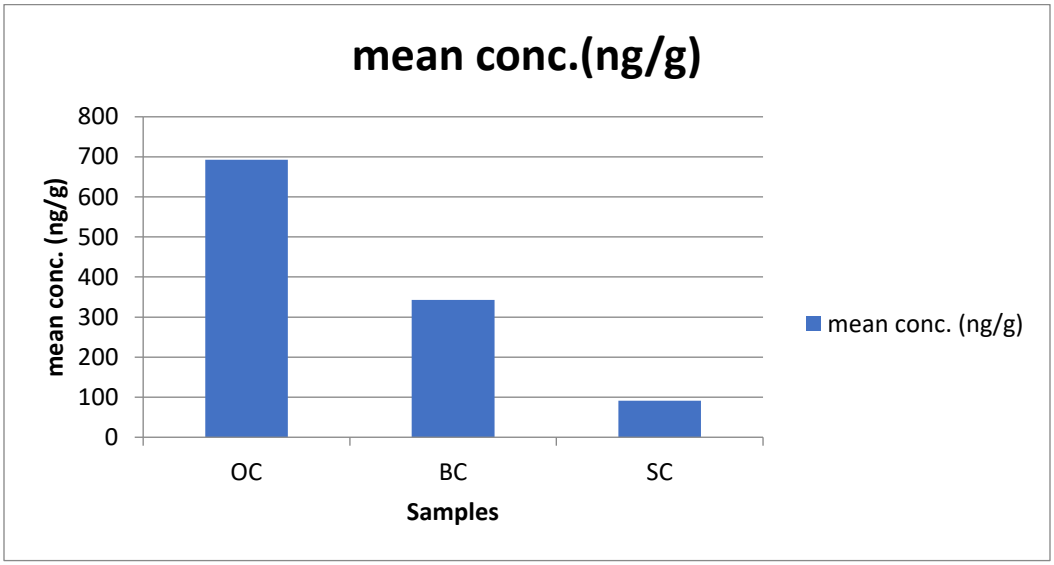

CONCLUSION

This research shows that chloramphenicol residue was identified in all of the samples, indicating that cattle farmers do not adhere to the withdrawal periods. It also revealed farmers using chloramphenicol in cows despite the EU, WHO banned it due to its possible carcinogenicity. This underlines the need for effective regulatory organizations to be set up to prevent drug addiction. Erythromycin levels in milk samples were negative. This does not rule out the potential that erythromycin was present in the samples at concentrations below the HPLC used in this study's detection limit. High linearity, sensitivity, precision and accuracy of high-performance liquid chromatography (HPLC) used, make it recommendable of analytical method for determining antibiotic residues in food matrices.

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