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Determination of the Levels of Chloramphenicol (CAP) and Tetracycline (TTC) Residues in Fresh and Fried Curdled Milk Collected From Ojo Market in Ibadan, Southwest Nigeria Using HPLC-UV

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Abstract

Antibiotics are substances that can be produced by a particular microorganism or synthesized either through synthetic or semi-synthetic processes, thereby inhibiting the development of alternatives. Microorganisms are essential bioactive and chemotherapeutic classes of compounds used for the prevention and treatment of bacterial infections in humans and animals. In veterinary medicines, antibiotics are generally used to improve or maintain the health of animals.

The aim of this study is to determine the levels of chloramphenicol (CAP) and tetracycline (TTC) residues in fresh and fried curdled milk collected from Ojo market in Ibadan using HPLC-UV as the detection method and also to determine how frying affects the concentration of these antibiotic residues in cuddled milk. For this purpose, the drug residue was recovered using solvent extraction method. The method of extraction involved the utilization of acetonitrile-water (90:10) and 0.1 mol/L Na_2EDTA solutions for the extraction process and cleaned up further by solid phase extraction process. This was centrifuged and supernatant was filtered, filtrate was taken for HPLC analysis. Blank samples and replicates were properly prepared to achieve a consistent result. The sample was spiked with 50 ppb for recovery studies.

The calibration plots for both analytes are linear with r^2 values of 0.994 and 0.995 respectively for chloramphenicol and tetracycline. The mobile phase utilized for chloramphenicol was; water:methanol: glacial acetic acid in the ratio; 55:45:0.1, while water:acetonitrile in the ratio 40:60 was utilized in the determination of tetracycline. New chloramphenicol concentrations in fresh cheese and fried cheese were found to be 7.33 16 ug/g and 0.1261 ug/g respectively, whereas the tetracycline concentrations in fresh cheese and fried cheese were identified as 2.5443 µg/g and 0.9682 µg/g, respectively.

This result shows that both antibiotics were heat-denatured but tetracycline is found to show more thermal stability than chloramphenicol. Chloramphenicol, which is withdrawn from most countries is still being used in Nigeria for therapeutic, prophylactic and growth promoting reasons. The amount of TTC (2.5443 µg - 0.9682 µg/g) exceeds the upper limit specified set by the European Union, which is 0.1 µg/g. Thus, it is advised that adequate monitoring strategies be directed towards controlling the usage of antibiotic drugs among cattle.

Keywords: Chloramphenicol (CAP); Tetracycline (TTC); Curdled Milk; HPLC-UV

Introduction

The word antibiotic implies "opposing life". This is derived from its Greek origins, (ανπ-) anti which means "against" and (βίος-) biotic which implies "life". Thus, antibiotics are widely utilized to refer to any substance used against microbes [35].

Antibiotics are chemicals either manufactured by one microorganism or synthetically or semi-synthetically produced which suppress the growth of other microorganisms. Similarly, they are important bioactive and chemotherapeutic classes of compounds produced by microbiological synthesis typically utilized for the prevention or treatment of most kinds of diseases caused by infectious agents encountered in human beings [35].

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Antibiotics are a class of medications that are utilized in the prevention and treatment of bacterial infections in humans and animals. They are low to medium molecular weight compounds with diverse chemical and biological activities. Antibiotics are predominantly utilized for chemotherapeutic and prophylactic treatment. Broadly in veterinary medicines, they are utilized for enhancing or preserving the health of animal species, and as feed additives to enhance growth and improve feed productiveness. A large amount of antibiotics in the form active pharmaceutical ingredients has been utilized in animal husbandry and in fish farming due to due to their high efficiency in growth promotion or disease control [11, 33]. Within the dairy sector, antibiotics have been utilized for over fifty years in dairy cow production for the treatment or prevention of diseases like the udder disease and also for the increased production of milk.

While antibiotics were initially manufactured for human use to treat antibacterial infections that are; unlikely to clear up without antibiotics, could infect others unless treated, or could take too take longer to clear without treatment, and pose a risk of more severe complications.

However, antimicrobial has been utilized for veterinary use nearly as quickly as they had been formulated for human medicine. They are incorporated into feed for growth promotion purposes, utilized for treatment of infections and are significant during the development of intensive methods of animal husbandry (Dairy farms). As a result, there are major hazards that may be presented by antibiotic residues in foods such as raw bulk milk (e.g., allergic effects, toxicity, carcinogenicity, resistant bacteria selection, interference with human normal flora, induce immunological response and inhibition of the starter culture).

Antibiotics have been utilized in the dairy sector for over half a century. It was used particularly in dairy cattle farming, to prevent or cure disease and to boost milk production or enhance feed efficiency. Research has revealed that this antibiotic makes its way into different dairy products like milk. Residual antibiotics in milk can seriously affect consumers' health eliciting allergic reactions and creating resistant strains. Milk contamination with antibiotics can also cause significant economic losses for producers and manufacturers of milk and milk products.

While antimicrobial medicines are helpful in the therapy of human infections, their presence in foods consumed by the public has negative public health consequences like drug resistance and hypersensitivity that could be life-threatening. The use of antibiotics therapy to treat and prevent control of udder infections in cows is an important part of mastitis control in most nations [35]. Due to extensive antibiotic use in the treatment of mastitis in dairy cattle, great efforts and issues have been geared towards the effective management and surveillance of antibiotics use in cow treatments to avoid raw milk contamination. The extensive antibiotic use has generated possible residue issues in milk and milk products that are consumed by the general public and due to the public health importance, milk and milk products contaminated with antibiotics above a specified residue levels, are not suitable for human consumption [35]. Extensive application of antibiotics in clinical practice has resulted in the presence of antibiotic residues in many food products of animal origin such as milk, egg, honey, and meat.

Antibiotics as Veterinary Drugs

Veterinary medicines are mostly administered to animals to treat diseases, protect their health and as a nutritional supplement. Drug residues in milk from animal are a major regulatory and health concern. They are primarily sulfa medication, sulfamethazine, and antibiotics such as penicillin and tetracycline. They are given orally as feed additives or directly by injection. Antibiotic use can lead to drug residues in the milk, particularly if not used in accordance with label instructions. The antibiotic residue in milk can lead to allergic reactions in sensitive people, slow down the growth of starter cultures in the manufacturing of cheese and other milk products, or suggest that the milk can come from an animal that has a severe infection [29]. Antibiotic residues find their way into the milk production chain at the farm level. Hence, producers need to understand the factors causing antibiotic residues in milk and how these residues can be prevented. In addition, the milk testing program must become a part of the quality control procedure based on the farm, quantifying the success of the industry to produce high-quality milk and not a regulatory program seeking defective products. The usage of antibiotics varies from country to country, within a country and between farms, based on policies. Additionally, systems employed to identify antibiotics in EU nations are formulated and executed by governments, enterprises, and farmers with numerous differences (Girma et al., 2014).

Materials and Method

The research was conducted at Ojo market, Ibadan, Oyo State, Nigeria, located in the rainy forest agro-ecological zone. Ojo falls between Jatitude 74672N. longitude 3.9122' E. The Fulani women who sell cheese (wara) as a means of lively hood are majorly found here. From this point, many of them go about to hawk their cheese (wara) for the general populace consumption in Ibadan.

Dairy Products and Milk

Milk is the secretion of the mammary glands of mammals used for feeding their offspring. Milk and other dairy products are significant sources of animal protein, which play key roles in Human growth and development. They are significant results of the Fulani agro-pastoral system and other peri-urban small holder systems of Nigeria.

West African Cheese

Cheese making is one of the oldest methods of preserving excess milk. There are different cheeses, the best-known being perhaps cheddar cheese. Cheese making is a major business worth billions of dollars in most industrialized countries. Cheeses are produced by the curdling of casein (the principal milk protein) either by an enzyme preparation, which is also known as rennet or an acid, typically lactic acid. Normal rennet used for industrial cheese-making has an enzyme, chymosin or rennin, which is derived from the abomasun (fourth stomach) of milk-fed calves aged 10 to 30 days. Industrial cheese is favored by calf rennet produce due to its limited proteolytic activity. Nonetheless, the global scarcity of calf rennet has given rise to the utilization of substitutes such as microbial rennets. The Fulani pastoralists have, since ancient times, used the aqueous extract from the leaves of Sodom Apple (Calotropis procera), known as Bomubomu' in Yoruba or Tumfafiya' in Hausa for the production of a soft cheese also called Warankasi in Nigeria and Woagachi in the Republic of Benin. This is a valuable source of animal protein, commonly utilized as a meat or fish substitute.

Preparation of Fried Cheese: 100 ml vegetable oil was poured

into a clean small frying pan. The frying pan containing the oil had been heated under controlled conditions. The cheese was added when the temperature had reached approximately 100°C. Stacked on top of one another in the boiling oil, the cheese was kept turning constantly till it became a slight brown in color. The cheese was drained off the oil and allowed to cool thereafter.

Sampling

Raw milk cheese was bought from Ojo market in Ibadan. The cheese was transported in an ice chest at a temperature of approximately -4°C to the laboratory. The cheese was divided equally into two parts, wrapped in aluminum foil and labeled properly with masking tape and permanent marker. A part was fried and the other half was left raw. Both parts were separated, crushed and later collected; subsequently kept at about -21°C until examination.

All glassware was cleaned by soaking in 3 M HNO overnight and then washed with detergent solution and then rinsed with distilled water. The glassware was dried in an oven at 105°C.

Reagents and Equipment Used

a. Disodium ethylene diAmineTetraAcetate (Na, EDTA)

b. HPLC Grade methanol

c. HPLC Grade acetonitrile

d. Purified Water

e. Centrifuge

f. Vortex Mixer

g. Sonicator

h. High-Performance Liquid Chromatograph

i. pH Meter,

J. Weighing Balance

k. Oven

l. C-18 Cartridge

m. Syringe Filter

Preparation of Reagents

Acetonitrile prepared in Water (ACN-HO) (90:10, v/v)

90 mL of acetonitrile was quantitatively transferred using a measuring cvlinder, to a 100 ml volumetric flask, thereafter, distilled water was added to make up the volume.

Preparation of 0.1 M Disodium Ethylene Diamine Tetraacetate (Na_2EDTA)

Approximately 37.2237 grams of Na_2EDTA salt was weighed on a calibrated analytical balance which was then dissolved in a 1-liter standard volumetric flask and made up to the calibration mark.

Preparation of ACN – H_20 (20:80 v/v)

Approximately 20 mL acetonitrile was accurately quantitatively transferred into a 100 mL standard flask and diluted to the mark using distilled water.

Sample Preparation

Extraction: The sample was homogenized and two grams of the homogenized sample was weighed into a 15 mL polypropylene centrifuge tube. The samples for the recovery study were spiked with 20 µL of the standard working solution, followed by continuous vortexing for 30 seconds and the tube allowed to remain at room temperature for 2 hours. Then, 7.5 mL of ACN-H_20 (90:10. v/v) and 0.5 mL of 0.1 molL-1 Na_2EDTA solutions were added to the sample. The sample was then homogenized for 135 seconds at 4,000 rpm, ultrasonically oscillated for 15 minutes, and centrifuged at 4,000 rpm at 4°C for 20 minutes (Wang et al. 2017).

Clean Up: Approximately 5 mL of supernatant was taken from extraction and was filtered through SPE cartridges which was prepared by weighing 100 g of alumina into a pyrex beaker and heated in the oven at 205° for about 16 hours. It was allowed to cool and 3 mL of distilled water was added and mixed by shaking for 10 mins. A weighed quantity of the alumina was packed carefully into a syringe tube and pre-treated with 3 mL of methanol and 3 mL of extraction mixture, before drying by evaporation at 45°C. The residue obtained was re-dissolved in 1 mL of ACN-H_20 (20:80, v/v) and centrifuged at 4.000 rpm for 36 minutes. The supernatant was passed through a PVDF syringe filter (pore size 0.2 µm), and filtrate was retained for HPLC-UV analysis (Wang et al., 2017).

Preparation of Standards

Tetracycline: A stock standard solution of tetracycline was prepared by dissolving 25 mg of the reference standard in 25 ml of methanol. 6 working standard solutions were then prepared by diluting the stock solution to 20 ppb, 50 ppb, 200 ppb, 300 ppb, and 500 ppb with methanol in 25 L standard flask. 20 µL of each working standard (in duplicates) was spiked into the HPLC; the retention time and respective peak areas were created. The peak of the analyte was found by comparing the retention time of the ratio of analyte in the sample to that of the standard.

Chloramphenicol: 25 mg of chloramphenicol standard was weighed and dissolved quantitatively in distilled water to prepare a stock solution of 1000 ppm. Six (6) working standards were also prepared by serial dilution of the standards. The standards were prepared in duplicate and 20 µL of each standard was introduced into the HPLC system, in which the retention time and respective peak areas were identified. The analyte's retention time was evaluated by the injection of 20 µL of each of the sample extracts. Peak areas were created. The analyte peak was verified by comparing the retention time of ratio of analyte in sample to that of standard.

Instrumentation

The HPLC System used was composed of a pump and a degasser, with a UV detector. The operating conditions were as follows: a CECIL CE4300 (CI8, 150 x 4.6mm, 5um), guard column (4.6mnn lD) an isocratic mobile phase of acetonitrile and water (60:40) for tetracycline and water, acetonitrile and glacial acetic acid (55:45:0.1) for chloramphenicol; pump flow l mL/min flow rate; injection volume was 20uL. The analytes were identified by comparing the chromatograms of the samples with that of the pure standards. The retention times of the the analytes in the chromatograms of standards were used as a direct point of comparison for the identification of the analytes (Table 1).

Validation of Results

Recovery Studies: 20 uL of 50 ppb mixed standard was added to the sample and the extraction, clean up and analysis was carried out as stated above. Percentage recovery was determined according to the following equation (Harris, 2001):

Table 1: Chromatographic Conditions.

Analyte	Mobile Phase	Conditions Used
Chloramphenicol	Watar:Methanol:Acetic acid 55:45:0.1M	Wavelength 272 nm Temperature 25°C Flow Rate 1 Ml/MIN
Tetracycline	Water:Acetonitrile 40:60	Wavelength 272 nm Temperature 25°C Flow Rate 1 Ml/MIN

% Recovery =Conc. of spiked Sample - Conc. of unspiked sample X 100 / Concentration of added standard.

Calibration Curves

The calibration plot of tetracycline and chloramphenicol was achieved by plotting mean peak areas derived from replicate analysis of their standard solutions versus their respective concentrations.

The limit of detection (LOD) and limit of quantification (LOQ) are calculated using the equations below;

LOD = 3 x standard deviation / Slope of calibration curve

LOQ = 10 x Standard deviation / Slope of calibration curve

Result and Discussion

Results

Chloramphenicol and Tetracycline in Cow Cheese: As obtained from the analysis, chloramphenicol and tetracycline were detected from both raw and fried cheese (Table 2).

Discussion

Analysis of Residual Antibiotic in Cheese: The method of analysis as applied in this study is as described by [53]. The samples were homogenized as it undergoes extraction in order to increase the overall surface area contact between the extraction solvent(s) and the sample leading to an improved isolation of the antibiotics residue from the matrices. An irreplaceable role in solvent extraction is played by the components of the extraction mixtures as both organic solvent and aqueous buffer have gained wide application in solvent extraction. One of the most commonly used organic solvent is acetonitrile because of its ability to precipitate proteins and extract compounds of interest. A high extraction efficiency and a very good deproteinization is a known ability exhibited by acetonitrile. Polar compounds are suitably extracted by aqueous solvents; several modified extraction procedures use the combination of water or aqueous buffer with organic solvents. Therefore, the mixture of acetonitrile and water was chosen as extraction solvent in this research.

In the extraction procedure, Na_2EDTA was added because antibiotics such as tetracycline possess a strong tendency to form complexes with metal ions in samples thereby resulting in low extraction efficiency. Na_2EDTA in the extraction mixture was used as a masking agent for the interfering metal ions. 0.5 mL of 0.1 mol L^{-1} Na_2EDTA was used for the extraction because it was found that excess Na_2EDTA may chelate with tetracycline [53].

Table 2: Concentration of Chloramphenicol and Tetracycline in Cheese.

	Mean Concentration of CAP		Mean Concentration of TTC	
SAMPLE ID	µg/mL	µg/g	µg/g	µg/mL
W1	9.1646	7.3316	3.1804	2.5243
F2	0.1576	0.1261	1.2102	0.9682

Key: ID – Identification; W1 – Raw Cheese (Wara); F2 – Fried Cheese (Wara); CAP – Chloramphenicol; TTC – Tetracycline

Solid phase extraction (SPE), employing alumina as the sorbent material, was utilized for the purification of the extract in order to remove interfering compounds which co-isolated with the analytes.

The research examines the level of chloramphenicol and tetracycline residues in the raw cheese which was found to be 7.3316 ug/g and 2.5443 ug/g respectively whereas in fried cheese, chloramphenicol and tetracycline contain the concentrations 0.1261 ug/g and 0,9682 ug/g respectively as presented in Table 1. Chloramphenicol was at a greater concentration than tetracycline in the cheese sample and this could mean that chloramphenicol is administered more to the animals than tetracycline.

Tetracycline Antibiotic Residue Concentration in the Sample: These values, which range from 0.9682 ppm in fried cheese to 2.5443 ppm in raw cheese, are higher than the levels of tetracycline residues that Tona and Olusola (2014) found to range from 0.0019 ppm to 0.0080 ppm in their analysis of milk and dairy products. The presence of the tetracycline antibiotic residue in the raw cheese sample might be due to various reasons, including the milking of animals a few hours after they had been treated with tetracycline antibiotics. Alternatively, it may be due to the administration of an over-the-counter dose of the antibiotic to these animals.

Despite the radical reduction in tetracycline concentration in the sample after frying, quite a large amount was still traceable. The heat-resistant characteristic of tetracycline is the reason the tetracycline antibiotic residues were stable in the cheese samples. This explains why complete denaturation through heat treatment is impossible. This fact has also been documented by [4, 27].

The European Union (EU) does not allow tetracycline residue in milk products above the 0.1 ppm Maximum Residue Limit (MRL) [17]. The tetracycline concentrations found in this study are above the maximum level set by the EU, making the cheese not fit for human consumption.

Chloramphenicol Antibiotic Residue levels in the sample: Chloramphenicol concentration in samples analyzed in this study ranged from 7.331 6 ppm (in fresh cheese) to 0.1261 ppm (fried cheese). In this observation, it was indicated that via heat effect, the chloramphenicol residues could be significantly denatured. This is similar to the discovery by Heshmati (2015) that chloramphenicol lacks thermal stability. Chloramphenicol, an erstwhile widely used veterinary medicine, has also been withdrawn from use in several countries; the findings of this research, however, indicate the drug's ongoing use in Nigeria for veterinary purposes.

Conclusion and Recommendations

Conclusion

In this work, chloramphenicol and tetracycline were detected in cheese. This is a pointer to the fact that antibiotic which has been phased out in most countries are still being used in Nigeria for therapeutic, prophylactic and growth promoting purposes. Chloramphenicol has been prohibited for use by WHO, FAO, EU, FDA due to its carcinogenicity which is a potential risk to human health.

The result of this work showed that TTC is present at a concentration exceeding the established maximum residue limit by the EU Regulatory Commission. This calls for concern as a result of its health effect. It is important to note that antibiotics are not dose dependent but rather depend on the level of sensitivity of

the individual to the drug. Therefore, the exposure of a sensitive individual to a very small concentration of this drug may have fatal effect.

This work also revealed that frying had little effect on the level of antibiotic drug residue detected in the sample. Therefore, when antibiotic residues occur in milk, their activities remain intact with partial reduction during pasteurization, frying or in the processing of milk. Failure to observe antibiotic withdrawal periods by cattle rearers in Ibadan and environ will likely expose consumers to products containing residues above tolerable limits.

Recommendations

1 There is need for the creation of awareness among farmers, consumers and policy makers on antimicrobial residue contamination of foods of animal origin and of the health hazards.

2. A national monitoring program of foods of animal origin is required in order to establish safe and tolerable limits.

3. Farmers and animal health workers should observe drug withdrawal periods at all times.

4. Socio-economic analysis of non-compliance would be required to understand the potential incentives that would lead to behavioral change among farmers.

5. Further research on the effect of temperature on antimicrobial residues should be carried out.

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