https://dx.doi.org/10.4314/dujopas.v9i3a.5
ISSN (Print): 2476-8316
ISSN (Online): 2635-3490
Dutse Journal of Pure and Applied Sciences (DUJOPAS), *Vol. 9 No. 3a September 2023*

Development and Validation of Reverse - Phase HPLC Method for Detection of Tetracycline Residue in Fresh Milk Samples

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Abstract
Dairy products are highly required by humans for growth, development and repairs as they are good sources of essential nutrients. Indiscriminate administration of veterinary drugs for prophylactic and therapeutic purposes could lead to the presence of their residues in dairy products such as milk. Reversed-phase high performance liquid chromatographic method with UV detection was developed and validated for detection of tetracycline in milk from five different samples. Mobile phase consisting of a mixture of deionized water, acetonitrile, monobasic potassium and acetic acid was delivered at a flow rate of 1.0 mL/min. The column used was Agilent ZORBAX ODS 4.6 X 150 mm 5µm.. The eluent was monitored by UV detection at 220 nm. Analysis was performed at 35^{o}C and the total run time was 20 minutes. Analytical parameters such as linearity, accuracy, precision and limit of detection were determined by validation procedure and found to be satisfactory. Calibration curve was linear over the concentration range of 0.01- 0.5 mg/mL with an R^2 of 0.9978. The limits of detection and limit of quantification were found to be 0.00023 mg/mL and 0.00110 mg/mL, respectively. The % RSD is found to be 3.62, 0.23 and 0.44 for 0.01, 0.1 and 0.5 mg/mL concentration with percentage recovery of 78.7 – 98.6 %. Generally, the developed method was found to be simple, rapid, precise and accurate for quality control of tetracycline in fresh milk samples.

Keywords: Tetracycline, HPLC, Validation, Milk analysis, Calibration

INTRODUCTION
Antibiotics are generally, considered as chemical substances which may possibly be compounds of antimicrobial, antibacterial, and antifungal that are able to destroy bacteria or inhibits their activity. Its global utilization to treat infections saved most military personnel that were injured during the war but their development implies implementation of a rigorous compliance of regulatory agencies (Bhalode *et al*., 2021; Sampat *et al*., 2022; Bungau *et al*., 2015). A considerable augment among the populace has recorded tremendous progress in use of antibiotics, which is generally due to specific activity alongside bacteria or fungi in animal hosts and, at same time, ability to increase growth rates and improve necessary feed efficiency in animal (Rehman *et al*., 2015). Their presence in environment is contributing to increase in

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number of multi-resistant bacteria, with subsequent serious implications for animal health (Jia *et al*., 2016; Angeles *et al*. 2023). Subsequently, many countries have imposed maximum residues limits for antibiotic in foodstuffs of animal, which vary in ppb - ppm range, depending on molecule and the food matrix. Some antibiotics are banned for animal use in some parts of world while still permissible in other countries. In this regard, sensitive analytical method is essential, for quantitative determination of antibiotics in food and environment. Electrochemical methods have the reward of allowing a reliable, fast and portable, in-field analysis to detect growing range of antibiotics residue in milk sample (Feier *et al*., 2017a,; Feier *et al*., 2017b; Stearns *et al*., 2017; Hsiao *et al*., 2018; Cui *et al*., 2023). Particular interest is occupied by electrochemical sensors based, which are current analytical devices that have the benefit of having high selectivity, quick detection (Meng *et al*., 2022; Abjean *et al*., 2023).

Tetracycline is widely administered to food producing animals due to their availability, ease of administration and broad spectrum antimicrobial activity as well as low cost (Gorter *et al*., 2015; Yu *et al*., 2020). Tetracycline antibiotics are produced by *Streptomyces* spp. One of the major drugs used for veterinary purposes are the group of Tetracyclines. They are widely used for treating animal infections (Chopra 2001; Vaughn *et al*., 2021). These groups are synthetic antibacterial agents commonly used for the treatment and prevention of infections in animals (Jayalakshmi *et al*., 2017; Martins *et al*.,. 2015; Al-Kuraishy., *et al*., 2021). They are also used for therapeutic purpose due to their low cost, broad spectrum and availability. Tetracyclines are amphoteric in nature with similar pka's, they form acids and bases. At extreme pHs they can be stable. They show an affinity for the formation of metal ion chelates. Thus, the misuse of tetracycline is hazardous to human health (Linton and Lange 1978). Subtherapeutic tetracyclines when wrongly administered lead to the development of resistant bacteria in the human body system. The organisms become resistant to tetracyclines and to other agents (Wilson and Wright., 1982; Chen *et al*., 2021). Gastrointestinal disturbances is another effect of the tetracyclines (Muriuki *et al*., 2001; Baker and Leyland., 1983), poorly foetal development (Cohlan, *et al*., 1963) and hypersensitivity (Idowu *et al*., 2010) and other toxic effects. The World Health Organization (WHO) together with the Food Agriculture Organization (FAO) have provided standards (Wegman *et al*., 2001) for acceptable daily intake and maximum residue limits in foods. For consumer's health protection, national regulatory agencies and international organizations such as Codex Alimentarius, European Commission (EC) and FDA established a strict regulations for TCs (Ngoc Do *et al*., 2020). The acceptable daily intake (ADI) for TC is 25 µg/kg bw/day (Food and Drug Administration, 2006; Hawser *et al*., 2011)

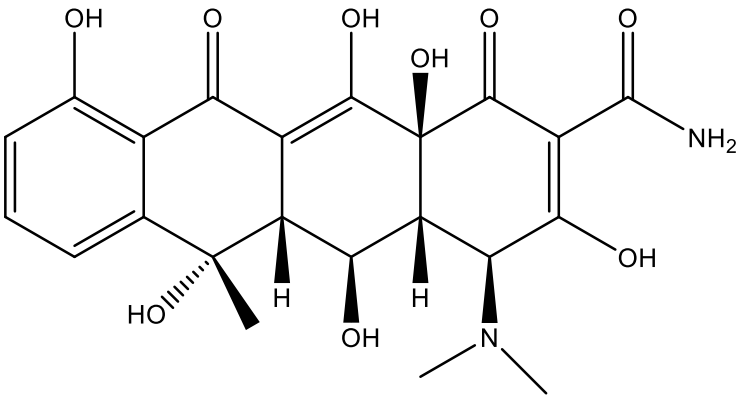

Figure 1: Structure of Tetracycline.

Determination of antibiotics in milk sample by high performance liquid chromatography (HPLC) is considered the most suitable quantification method chosen for validation. It has high sensitivity, specificity and reproducibility, and capable of measuring a wide range of concentrations of this antibiotic, besides its low cost (Stoob *et al*., 2005). Few analytical techniques have been described for assay of tetracycline in milk samples (Mauro *et al*., 2019; Totoli *et al*., 2018; Zhang *et al*., 2019; Ibrahim and Nasir 2014; Cestaro *et al*., 2023). However, they are considered time-consuming and of restricted application (Goncalves *et al*., 2008). Chromatographic analysis, especially high performance liquid chromatography (HPLC), is the most utilized strategy these days for determination of antibiotics due to its specificity, sensitivity, efficiency and reproducibility. Reverse phase chromatography (RPC) is the type that operates on the principle of hydrophobic interactions, which result from repulsive forces between a polar eluent, the relatively non-polar analyte, and the non-polar stationary phase. There are a few detection conditions related with HPLC for the quantification of tetracycline including spectrophotometry (HPLC-UV) and mass spectrometry (HPLC-MS). High performance liquid chromatography (HPLC) as a sensitive and specific method for the analysis of tetracycline was used for method development. The method developed in this work is a sensitive analytical method based on a highly specific and selective chromatographic technique. This method is simple compared to those previously used in literatures. The aim of developing the method is to determine tetracycline residue in milk samples.

MATERIALS AND METHOD

The materials and chemicals used in this experiment were of high purity. Tetracycline was purchased from Sigma Aldrich. To ensure consistent results, HPLC grades acetonitrile from Fischer Scientific UK was used. Other solvents include potassium dihydrogen phosphate, R and M chemicals, triethylamine, Sigma Aldrich, tetrahydrofuran R and M chemicals, were analytical grade chemicals, prepared and filtered using Nylon 66 membrane filters.

Instrumentation

The HPLC analysis was performed on a Agilent 1200 Series LC, equipped with a Quaternary Pump solvent delivery system capable of mixing four different solvents at the same time, a degasser, a column heater, and an auto sampler. Samples were detected with variable wavelength detector and data was processed using Agilent ChemStation Software. The HPLC column used throughout this experiment was Agilent ZORBAX ODS 4.6 X 150mm 5µm.

Analytical assays

Milk samples were analyzed for tetracycline using a validated high-performance liquid chromatography (HPLC) method with UV detection at 220 nm. Milk samples were extracted at room temperature using acetonitrile. Then, 5 µl volume was injected to HPLC analysis under optimum separation conditions. Mobile phase consisting of a mixture of deionized water, acetonitrile, monobasic potassium, 1N acidified with acetic acid as ratio (909:80:10:1) was delivered at a flow rate of 1.0 mL/min with UV detection at 220 nm. Analysis was performed at 35 ^{o}C and the total run time was 20 min. In order to obtain valid results, the HPLC method for the antibiotic was based on the U.S Pharmacopeia (USP 29).

RESULTS AND DISCUSSION

Linearity and range of concentration of tetracycline

For determining the linearity, a series of solutions with different standard tetracycline concentration range of 0.01 – 0.5 mg/mL were prepared by simple dilution of stock solutions.

The calibration graph for tetracycline in Fig. 1 was obtained by plotting the area under peak versus known concentrations in mg/mL. The linear equation of tetracycline obtained is

$y = 27355x + 945.9$ with R^2 of 0.9978.

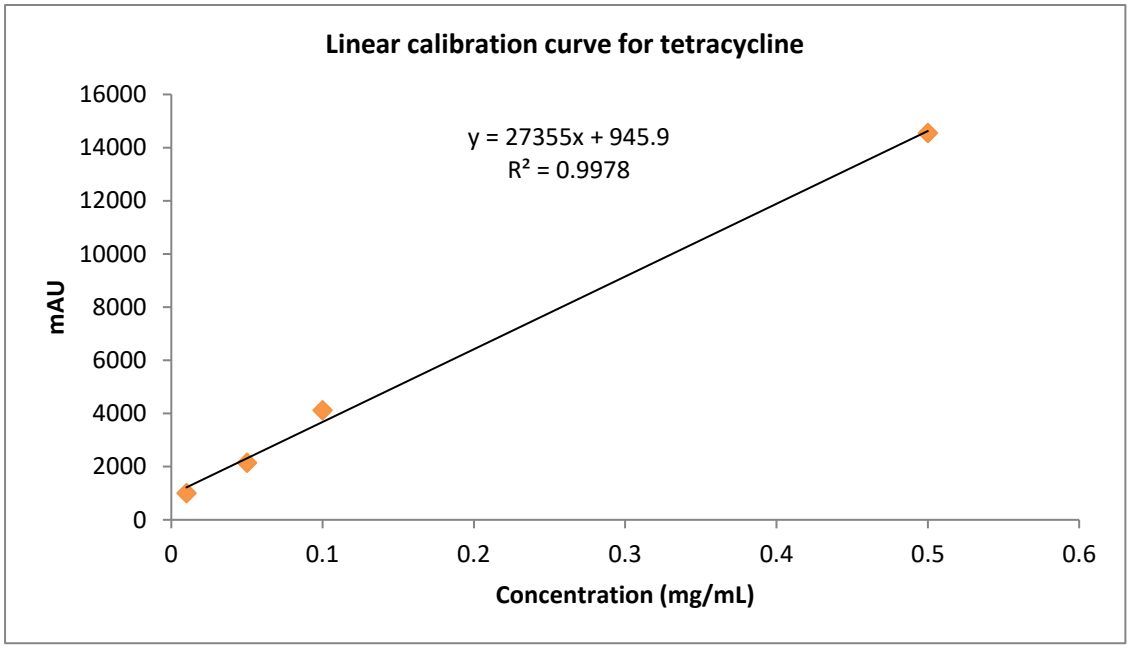

Figure 1: The Plot of Mean Peak Area versus Concentration of Tetracycline

The r^2 value of 0.9978 shows a good regression line in the range of the explored concentrations (0.01 – 0.5). It also indicates the accuracy of the curve, hence the more accurately our curve represents the detector response. The r^2 value represents the goodness of fit of a model.

Specificity towards detection of tetracycline

Specificity is a chromatographic design system to analyze active component, which is the separation of components of tetracycline which provides retention time and selectivity as in Fig. 2. The retention time of concentrations between 0.01 to 0.5 mg/mL for tetracycline is about 3 minutes with good peaks of their respective concentration, in which solvent peak elutes at about 1 minute and with some impurities before and after tetracycline elution with carry over.

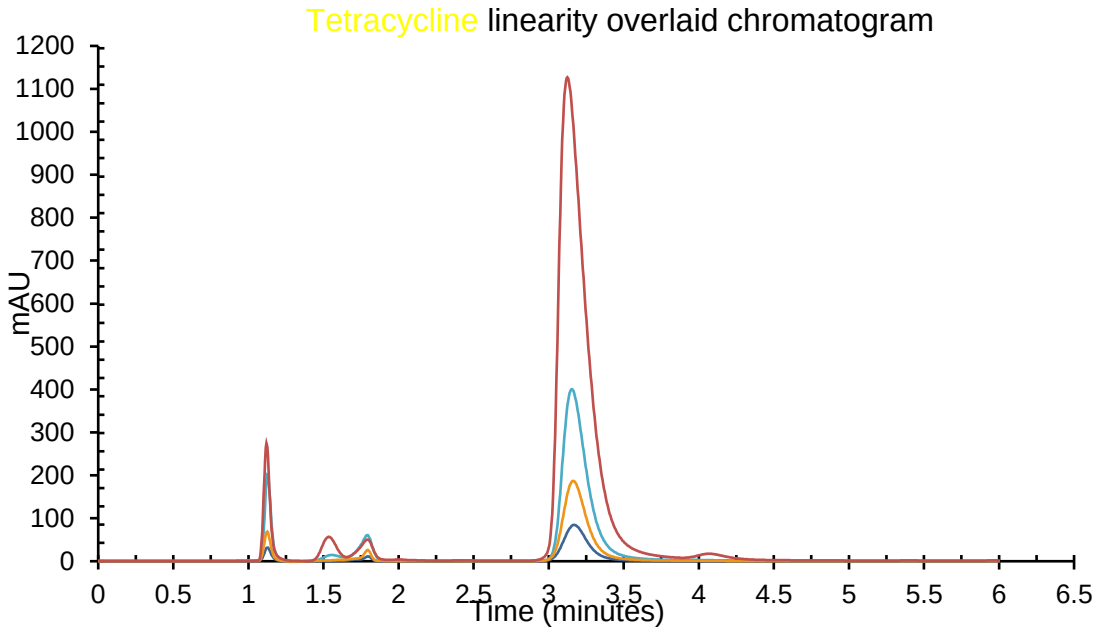

Figure 2: Chromatogram of Linearity range for the study

Accuracy

The accuracy was assessed based on three level of concentration 0.01, 0.1 and 0.5 mg/mL value. Triplicate run was carried out for tetracycline with relative standard deviation (RSD) values of 3.62, 0.23 and 0.44 % respectively as presented in Table 1. The results showed that the highest %RSD (3.63) was found in the first concentration level (0.01mg/mL).

Table 1: Accuracy level for tetracycline

Concentration	0.01 mg/ml	0.1mg/ml	0.5 mg/ml
Run 1	1047.17	4674.15	14599.50
Run 2	982.69	4660.93	14584.20
Run 3	987.08	4652.64	14482.30
Mean	1005.65	4662.57	14555.33
Std Deviation	36.02719	10.84875	63.70968
% RSD	3.62	0.23	0.44

Repeatability and recovery study of tetracycline

The repeatability study for tetracycline in Table 2 was performed which recorded the %RSD values between 0.44 – 3.58 %. The recovery study of tetracycline were performed by spiking known concentrations of tetracycline as shown in Table 2 with recovery between 78.7 to 98.4%, which indicates that the proposed method for determining tetracycline using this method is quite satisfactory with respect to the procedure and parameters obtained.

Table 2: Repeatability and Recovery study for tetracycline

Level Spiked	0.01 mg/mL	0.1 mg/mL	0.5mg/mL
Tetracycline	Recovered Conc.	Recovered Conc.	Recovered Conc.
run 1	0.0082	0.0790	0.493
run 2	0.0080	0.0780	0.493
run 3	0.0080	0.0790	0.490
Mean	0.0081	0.0787	0.492
%Recovery	81	78.7	98.6

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Limit of detection (LOD) and limit of quantitation (LOQ) of tetracycline

The LOD is the lowest amount of analyte which can be detected; the limit of detection of tetracycline is calculated to be 0.00023 mg/mL while LOQ of tetracycline is calculated to be 0.00110 mg/mL. This implied that the method was able to detect the samples at relatively low level.

CONCLUSION

The study aimed at determination of tetracycline in bovine milks of different species. The developed method proved to be simple, specific, accurate and with good linearity for determining tetracycline. The accuracy of the method was validated by mean percentage recovery which was confirmed to be in acceptable range.

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