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## Investigation of tetracycline residues in poultry meat samples from Dhaka city by high-performance liquid chromatography

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### ABSTRACT

The objective of the study was to quantify tetracycline (TCs) i.e., oxytetracycline (OTC), tetracycline (TC) and chlortetracycline (CTC) residues in thirty poultry meat samples (n = 30) collected from the local market and super shop around the Dhaka University campus during May 2019 to January 2020. Three samples were collected from each of ten locations. All samples were extracted by Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) method. Samples were analyzed by reversed-phase High-Performance Liquid Chromatograph equipped with Photo Diode Array Detector (LC-PDA) and a reported method was validated with good linear correlation coefficients of standards and matrix-matched calibration curves with  $r^2=1.00$ , 0.99, 0.99 and  $r^2=0.99$ , 0.99, 0.99 in the linearity range of 0-10  $\mu\text{g}/\text{kg}$  for OTC, TC and CTC, respectively. The limit of detection (LOD) and limit of quantification (LOQ) for OTC, TC, and CTC were 1.05, 1.17, and 1.09  $\mu\text{g}/\text{kg}$  and 3.15, 3.51 and 3.27  $\mu\text{g}/\text{kg}$ , respectively. Accuracy that is expressed by the recovery percentages were calculated at two different concentrations (2.5 and 5  $\mu\text{g}/\text{kg}$ ) were 91 and 100%, 102 and 100%, and 106 and 100% for OTC, TC, and CTC, respectively. Intra-day (n=3) and inter-day (n=9, 3 days) precision data were under 10% for all sample matrixes. Standard deviations were calculated  $\pm 0.06$ ,  $\pm 0.11$  and  $\pm 0.03$  and precision (expressed by RSD%) were found 5.57, 9.14 and 2.35%, respectively for OTC, TC and CTC. The HPLC-PDA method is affordable for screening of large number meat samples for residual antibiotics in biological matrices by any laboratories. The method is also cheaper in comparison with LC-MS/MS. Analysis of real 30 poultry meat samples showed that the tetracyclines residues were below the quantification limit in all samples.

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### 1. Introduction

Antibiotics are chemical substances derived from a natural, semi-synthetic or synthetic way that affect antibacterial activity by killing or inhibiting the growth of bacterial pathogens (Nita, 2007). Antibiotics are classified according to the mechanism of action (inhibitors of protein synthesis, membrane function, anti-metabolites, cell wall synthesis, and nucleic acid synthesis), on basis of a range of effectiveness (bactericidal or bacteriostatic), range of working efficiency (narrow or broad spectrum) and according to chemical structure (Darwish et al., 2013). They are administered parenterally or intravenously, topically, and orally (Geidam et al., 2009). Oxytetracycline (OTC), tetracycline (TC), and chlortetracycline (CTC) are the most widely used chemicals in the group of broad-spectrum tetracycline antibiotics and chlortetracycline was the first tetracycline antibiotic to be discovered in 1948 (Nelson et al., 2011). The basic structure of a tetracycline consists of a hydro-naphthalene backbone containing four fused rings (Ng et al., 2003) (Fig. 1, Table 1).

Tetracyclines are given to animals for the prevention and treatment of particular diseases as well as enhanced growth and imposed for human consumption. Animals grow faster and healthier due to the administration of these antibiotics. Acquisition of resistance by microorganisms can occur

because of the long-term administration of antimicrobial substances and the presence of residual antibiotics in the edible tissues of the animals, as well as in the environment i.e., soil and water. Therefore, the controlled use of antibiotics in veterinary medicine is an important matter in protecting the health of animals and consumers (Macarov et al., 2012; Chopra et al., 2001). But illegal, inappropriate, insensible, and uncontrolled usage of antibiotics in poultry causes residues in food of animal origin. Resistant bacteria populations increase and accumulate in various organs and tissues, particularly in the liver and kidneys as a result of insensible and unrestrained utilization of antibiotics (Doyle, 2006). Accordingly, if the withdrawal period is not successful, animals exposed to chemical substances may leave residues in their carcasses at the time of slaughter (Vragović et al., 2011). Nowadays, the existence of antibiotic residues in foods of animal origin has been a serious concern in the trading world. Antibiotics used in food animals can cause health hazards due to their secretion in edible animal tissues in trace amounts (Menkem et al., 2018). The presence of antibiotics may cause several adverse health effects to humans, like tissue damage, gastrointestinal disturbance, neurological disorders, and hypersensitivity (Ramatla et al., 2017). Bangladesh Food Safety Authority (BFSA, 2013) has set the maximum residue limit (MRL) values as 200, 600, 1200  $\mu\text{g}/\text{kg}$  for tetracyclines in muscles, liver

and kidney of poultry. The maximum residue level of TC residues in animal products set by *Codex Alimentarius Commission* to be 200 µg/kg in muscle, 600 µg/kg in the liver, 1200 µg/kg in fat and kidney (FAO/WHO Expert Committee on Food Additives, 2004) while European Union regulated as 100, 300 and 600 µg/kg, respectively in these organs to protect human health in muscle for poultry meat (Council Regulation (EEC) No. 2377/90/EC, 1990). Several analytical techniques including capillary electrophoresis (CE) (Nozal et al., 2004; Hernandez et al., 2000; Hernandez et al., 2002), high-performance liquid chromatography (HPLC) (Biswas et al., 2007; Blanchflower et al., 1997; Charlet et al., 2003; Zhou et al., 2009; Koesukwiwat et al., 2007; Tong et al., 2009; Cinquina et al., 2003; Tavakoli et al., 2003) with UV, PDA and fluorescence detection (Patyra et al., 2014; Esponda et al., 2009; Schneider et al., 2007; Lu et al., 2004; Grandos et al., 2005), and liquid chromatography-electrospray tandem mass spectrometry (Mou et al., 2021; Jia et al., 2009; Lykkeberg et al., 2004; Guoa et al., 2016; Elkhabeer et al., 2020) are available for determination of residual TCs in different biological matrices. Bahmani et al. (2020) reported the presence of OTC and TC residues in four poultry meat samples ranging 67.5-425.3 and 91.2-252.3 µg/kg and Ibrahim et al. (2015) detected OTC residues in three tested poultry meat samples ranging 150-500 µg/kg using HPLC-UV method. As food safety is a burning issue in Bangladesh, the determination and quantification of tetracycline in edible tissues of poultry will help BfSA to ensure safe food for the public health. The present study describes a reported method for the validation, qualitative and quantitative determination of three tetracyclines namely tetracycline (TC), oxytetracycline (OTC) and chlortetracycline (CTC) in poultry meat samples.

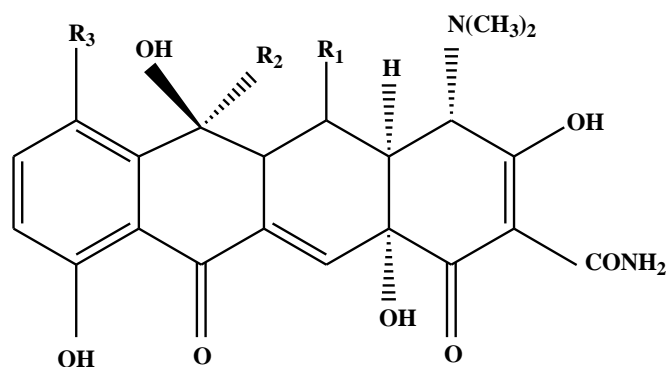


Fig. 1. Chemical structure of tetracycline

Table 1. Value of R<sub>1</sub>, R<sub>2</sub> and R<sub>3</sub> of TCs structure

Tetracycline	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>
TC	H	CH <sub>3</sub>	H
OTC	OH	CH <sub>3</sub>	H
CTC	H	CH <sub>3</sub>	Cl

Note: R<sub>1</sub>, R<sub>2</sub> and R<sub>3</sub> are functional groups of TCs

## 2. Materials and method

### 2.1. Sampling

A total of thirty poultry meat samples (n=30) were purchased from different super shops and local markets of Dhaka city and a control sample free from antibiotics was collected from the village. Three samples were collected from each of ten locations. Approximately, all samples were weighted 900-1000 g. The homogenous portion from muscles (thigh and breast) poultry samples obtained from each carcass were stored at -20 °C until analysis.

### 2.2. Chemicals and reagents

Oxytetracycline hydrochloride, tetracycline, and chlortetracycline hydrochloride (Sigma-Aldrich, Germany) were used to prepare standard solutions. HPLC grade acetonitrile (ACN) and methanol (MeOH), analytical grade C-18, primary secondary amine (PSA), oxalic acid (dehydrate), magnesium sulphate (MgSO<sub>4</sub>), and sodium chloride (NaCl) were purchased from Sigma-Aldrich, Germany. Deionized water used for HPLC was obtained from the Mill-Q System (Denmark & USA).

### 2.3. Instruments

A reversed-phase High-Performance Liquid Chromatograph (HPLC; RF 1200, Prominence, Shimadzu) equipped with Photo Diode Array Detector (PDA; SPD-M20A Prominence) connected with a Rheodyne injector (20 µL sample loop) was used for the analysis of tetracyclines. A C-18 column (Luna; 250 × 4.60 mm; particle size 5µm) kept in an oven at ambient temperature was used for analysis. The analytical balance (Type ATY124, Shimadzu), homogenizer, sonicator (Hwashin tech. com.) centrifuge machine (Sigma, 2-16P), and vortex mixer (REAX 2000) were used for sample preparation.

### 2.4. Preparation of standard solution

Stock Solutions of each TCs standard (oxytetracycline, tetracycline, and chlortetracycline) were prepared by dissolving 10 g of compound in 10 mL MeOH to obtain a final concentration of 1.0 mg/mL. The stock solution was wrapped with aluminum foil paper to prevent photodegradation and stored at -20 °C. The stock solution was diluted with MeOH to prepare required standard solutions and that were prepared daily when needed.

### 2.5. Fortification of samples

Poultry meat samples were fortified with a mixture of TCs standard at two spiking levels of 2.5 and 5 µg/kg.

### 2.6. Extraction and clean up procedure

Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) method was used for sample preparation. According to this method, poultry meat samples (10 g) were taken in a falcon tube. At first, 10 mL deionized water and 10 mL ACN were added to the samples. Then, meat samples were vortexed for 1 min, and 4 g magnesium sulphate (MgSO<sub>4</sub>) and 1 g sodium chloride (NaCl) were added in each sample and again vortexed for 1 min. Finally, centrifuged at 4000 rpm for 10 min. The clean-up procedures were performed by transferring 2 mL supernatant in a screw-capped test tube and adding 150 mg PSA (Primary Secondary Amine) and 250 mg C-18. Then vortexed for 1 min and centrifuged at 2000 rpm for 4 min.

### 2.7. HPLC analysis for TCs

The level of tetracyclines in extracted samples was determined using HPLC according to a reported method. Briefly, mobile phase an isocratic elution using 0.01 M oxalic acid buffer (a), ACN (b), MeOH (c) was applied by volume with 70:20:10 (v/v/v) for 15 min. The flow rate of the mobile phase was 1.0 mL/min and the column oven temperature was set up at 40 °C. The injection volume of the standard or sample was 20 µL. TCs were detected at 360 and 375 nm using a Photodiode Array Detector (PDA).

### 2.8. Method validation

#### 2.8.1. Linearity and sensitivity

This study was expanded to test the validity of a reported method. Linearity was assessed by calibration curve in the range of 0-10 µg/kg (0, 1.0, 2.5, 5.0 and 10µg/kg) for OTC, TC and CTC standard solutions at five points (Fig. 2) with triplicate

analysis (Table 3) and chromatogram of TCs standard were shown in Fig. 3.

The response of the PDA detector was linear and highly correlated with the amounts of TCs injected, where the enumerated correlation coefficient ( $r^2$ ) ranged from 0.99 to 1.00 and each TC standard had its linear equation. The sensitivity of the method is ordinarily explained by the slope of the analytical calibration curve. The sensitivity of this reported method was found to be 1.05, 1.17 and 1.09  $\mu\text{g}/\text{kg}$  for OTC, TC and CTC, respectively (Table 3). The calibration curves of standard compounds were drawn using peak area versus concentration following the linear least squares regression procedure. The accuracy is revealed as the relative standard deviation (RSD%) of the slope of the curves.

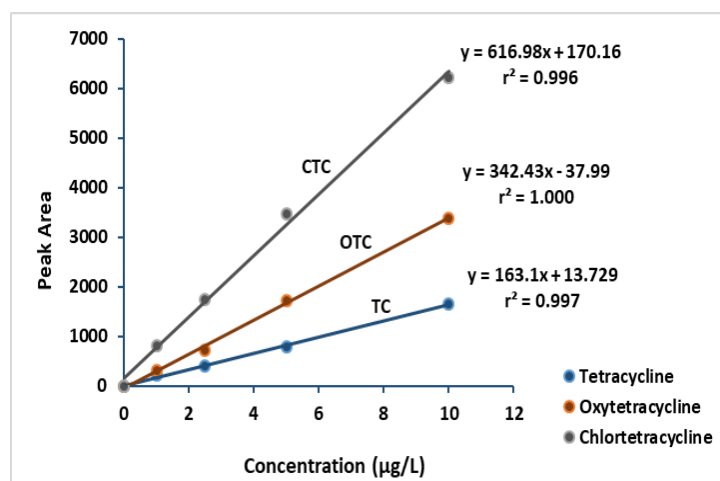


Fig. 2. Calibration curve for OTC, TC, and CTC standards

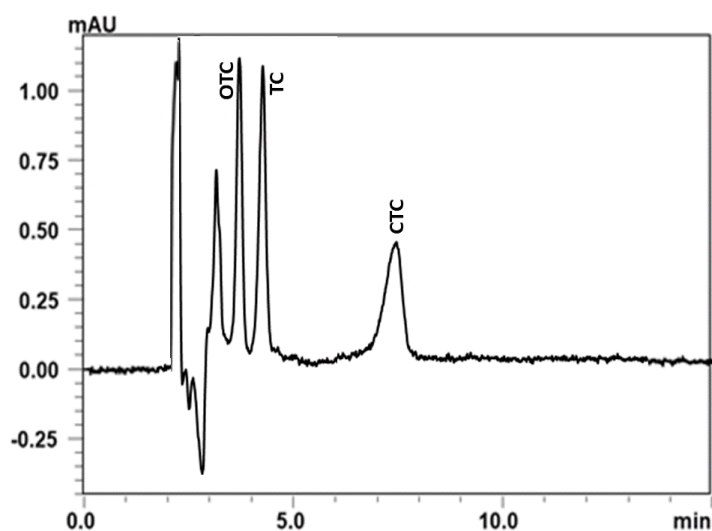


Fig. 3. Chromatogram of TC standards

### 2.8.2. Accuracy and precision

Accuracy is a measurement of the methodical deviation of the results from the true value. It is shown as the percentage of recovery while precision is the variability of the results narrated by the relative standard deviation (RSD%) of a set of replicate results. RSD (%) is related to the error of within-laboratory of a method. Recovery and precision (RSD %) were measured for five replicate analyses for both intra-day ( $n = 3$ ) and inter-day ( $n=9$ , 3 days) experiments. Recovery was determined

by comparison of response for samples spiked into sample matrix prior to sample preparation with response for spiked samples after all sample preparation steps. Therefore, any superficial recovery loss resulting from matrix suppression was eliminated from the recovery calculation. Poultry meat samples were fortified at two different concentrations (2.5 and 5  $\mu\text{g}/\text{kg}$ ), respectively in three replicates for measuring recoveries. The all-up precision ranged from 2.35 – 9.14 (RSD %) (Table 3) for poultry meat (muscles from breast & thigh). Although the acquired recovery values of all analytes in different samples within the Association of Official Analytical Chemists (AOAC approved on 19 Dec 2002 and used by ISO 17025) acceptable range for trace analysis; 60–120% of the values of OTC, TC, and CTC of poultry meat samples. The relative standard deviation (RSD) was lower than 10% than compliance Codex Alimentarius commission. For both inter-day and intra-day experiments, the RSD was under 10%. A method is considered as validated when RSD values are optimal.

### 2.8.3. Limit of detection (LOD) and limit of quantitation (LOQ)

The limit of detection (LOD) is calculated by three times of signal to noise ratio. LOD is the lowest concentration of an analyte in a sample. The limit of quantitation (LOQ) is the lowest amount that can be quantified within allowable accuracy and precision at the signal-to-noise ratio of 10. Both LOD and LOQ were assessed and the acquired data were given in Table 3. It was observed that the LOD of the proposed method was 1.05, 1.17, and 1.09  $\mu\text{g}/\text{kg}$  for OTC, TC, and CTC respectively. The similar values of LOQ were 3.15, 3.51, and 3.27  $\mu\text{g}/\text{kg}$ .

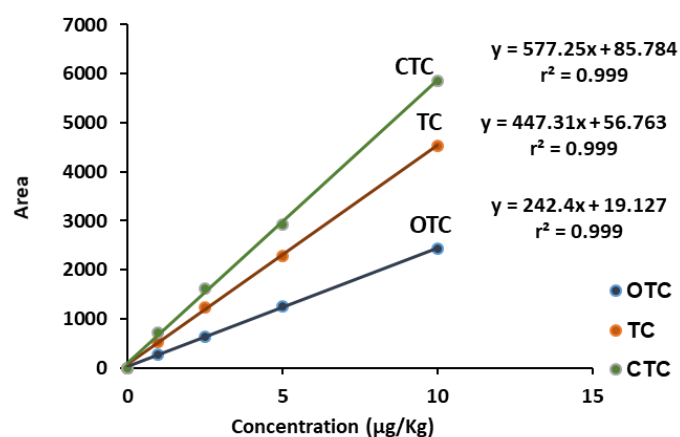


Fig. 4. Matrix-matched calibration curve for OTC, TC, and CTC

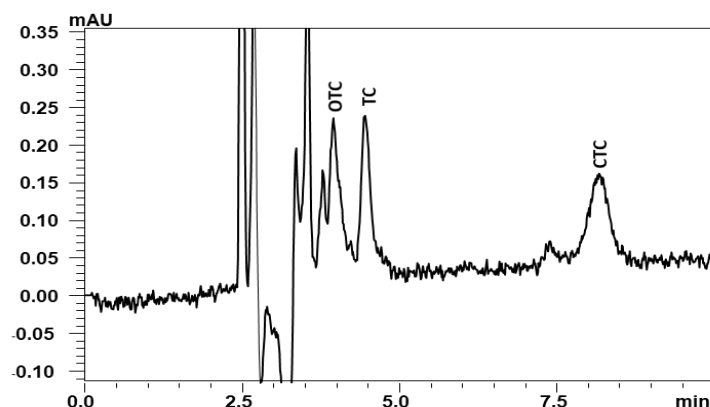


Fig. 5. Chromatogram of TC standards at spiking level 5  $\mu\text{g}/\text{kg}$

### 3. Results and discussion

The residual tetracycline (OTC, TC, and CTC) in poultry meat samples were analyzed by reversed-phase HPLC with a Photo Diode Array detector (PDA). TCs standard solution was separated on the C<sub>18</sub> column at two wavelengths, i.e., 360 and 375 nm. The average retention time was 3.8, 4.30, and 7.8 min, respectively for OTC, TC, and CTC. Linear calibration curves were produced for each standard OTC, TC, and CTC in the range of 0-10 µg/L, and the correlation coefficient, ( $r^2$ ) ranging from 0.99-1.00 was found for the standard.

The matrix match calibration curves were also carried out and a similar correlation coefficient was found which indicated that the matrix did not affect the analysis of OTC, TC, and CTC in poultry meat samples (Fig. 5). The value of the correlation coefficient obtained for each calibration curve showed that the correlation between peak area and concentration was excellent.

The LOD and LOQ values for OTC, TC, and CTC were 1.05, 1.17, and 1.09 µg/kg and 3.15, 3.51 & 3.27 µg/kg, respectively. Triplicate (n=3) recovery experiments for OTC, TC, and CTC were done by fortification of the control matrix (poultry from the village) at two fortified levels 2.5 µg/kg and 5 µg/kg. The average percent

recoveries were 91 and 100%, 102 and 100%, and 106 and 100% for OTC, TC, and CTC, respectively with the repeatability (RSD %) of 5.57, 9.14, and 2.35%, respectively. Thirty poultry meat samples were analyzed. However, residual tetracyclines in all samples were found below the detection limit (Fig. 6, Fig. 7).

Food safety is of great significance and a global issue to consumers all over the world (Shahbazi et al., 2015). Nowadays, antibiotic drugs are generally used to distribute three purposes in poultry in the livestock, firstly animals are administered with high doses of antibiotics for relatively shorter periods as therapeutic use, secondly, prophylactic use which includes disclosure of animals with medium dose of antimicrobials for long durations, thirdly the growth enhancement that is sub-therapeutic doses of antibiotics. As an example, it was said that 10 or 100 times less than therapeutic doses are given for a very long time or throughout the entire life of the animals (Marshall et al., 2011; Chowdhury et al., 2009). Several types of antimicrobial drugs were reported to be commonly used at the farmers' level belonging to most of the tetracycline and sulfonamides group (Milagro et al., 2008). Different groups of drugs have different withdrawal periods and their residual action in the animal body acts at different times (Khatun et al., 2018).

**Table 2.** Slope, Intercept and Correlation coefficient ( $r^2$ ) of both TCs standard and matrix-match TCs

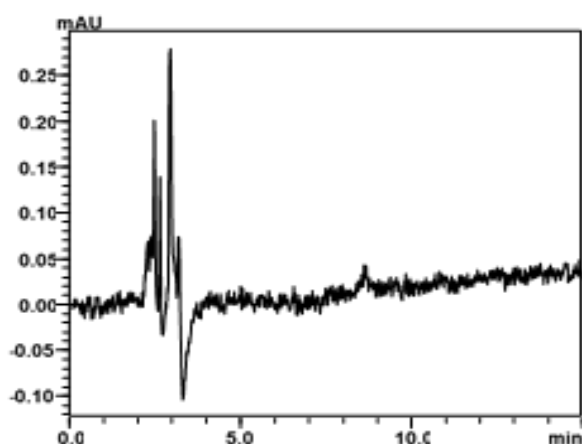
Antibiotics	Slope standard TCs	Intercept standard TCs	Correlation coefficient ( $r^2$ )	Slope (matrix-match)	Intercept (matrix-match)	Correlation coefficient ( $r^2$ ) (matrix-match)
OTC	342.43	-37.99	1.00	242.40	19.127	0.99
TC	161.10	13.73	0.99	447.31	51-6.763	0.99
CTC	616.98	170.16	0.99	577.25	85.78	0.99

Note:  $r^2$ = correlation coefficient; OTC=Oxytetracycline; TC= Tetracycline; CTC= Chlortetracycline

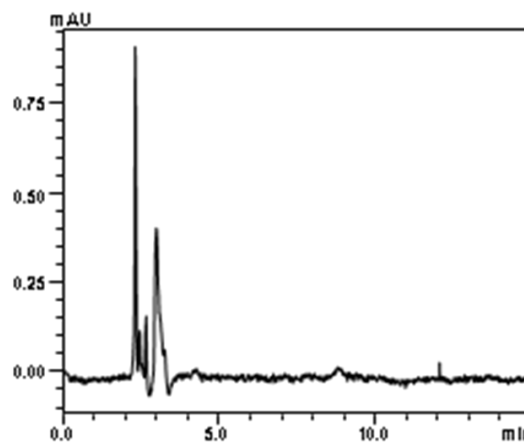
**Table 3.** Fortified level, mean recovery, average, SD, RSD, LOD, and LOQ of TCs

Antibiotics	Fortified level (µg/kg)	Mean Recovery (%) Inter-day (n=3 Days)	Average±SD	RSD (%)	LOD (µg/kg) (3:1)	LOQ (µg/kg) (10:1)
OTC	2.5 & 5	91 & 100	1.05 ± 0.06	5.57	1.05	3.15
TC		102 & 100	1.17 ± 0.11	9.14	1.17	3.51
CTC		106 & 100	1.09 ± 0.03	2.35	1.09	3.27

SD=standard deviation; RSD=relative standard deviation; LOD=limit of detection; LOQ=limit of quantitation



**Fig. 6.** Reagent Blank



**Fig. 7.** Poultry meat sample without containing any TCs residue

The presence of residual tetracycline in poultry meat and organs higher than the maximum residue limit was reported in the literature due to improper use of antibiotics by producers which is a great concern to the consumers and harmful for public health globally (Bahmani et al., 2020; Arslanbas et al., 2018; Shahbazi et al., 2015; Ibrahim et al., 2015). However, the present study revealed that poultry meat samples are free of residual tetracyclines

and safe for consumption which indicated that poultry farmers maintain the proper doses and withdrawal period of the antibiotics. The residual antibiotics may exit from treated animals by excretion and enter into the environment as a result, they were not detected in the present study (Aga et al., 2003). Therefore, further research on a large scale and other food matrices are necessary to ensure safe food for consumers in Bangladesh.



#### 4. Conclusion

Tetracycline antibiotics have been used globally for the treatment of bacterial infections in livestock. Some antibiotics like chloramphenicol and nitrofurantoin have been banned in many countries for livestock (Mou et al., 2021). A few researches were performed by Thin Layer Chromatography for the detection of residual antibiotics in Bangladesh. Sarker et al. (2018) reported the percentage of OTC residues 74, 47 and 36% in liver, thigh muscles and breast muscles. But adequate research in Bangladesh has been done by HPLC method due to insufficient facilities in all research laboratories, though that is an advanced and more sensitive method than TLC. The HPLC-PDA is an efficient, sensitive, and reliable method for the analysis of tetracyclines in poultry meat samples. The analyzed samples were free of residual tetracyclines and safe for consumers.

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#### Conflict of interest

The authors declare no conflict of interest.

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