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Quantitative Analysis of Residual Tetracyclines in Buffalo Meat using RP-HPLC

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ABSTRACT

A liquid chromatographic method is described for the simultaneous quantitative measurement of oxytetracycline and chlortetracycline in buffalo meat. After extraction of the meat samples using Mcllvaine buffer – EDTA solution and SPE clean up, the analysis of the antimicrobials was carried out using a reverse phase HPLC using UV detector. The chromatographic separation was accomplished with mobile phase consisting of 0.1M oxalic acid, methanol and acetonitrile (70:15:15, v/v) in an isocratic elution mode. The volume of injection and flow rate of the mobile were 1.5 ml/min and 50 µl respectively.UV detector was operated at a wavelength 360nm. Linearity, recovery, selectivity, limit of detection, limit of quantification was evaluated in buffalo meat matrix at drug concentrations ranging from 25-1000 ng/g. Mean extraction recoveries of oxytetracycline and chlortetracycline were in the range of 90-97 %. The limits of quantification for oxytetracycline and chlortetracycline were 38.30 and 36.63 µg/kg, respectively. The proposed RP-HPLC method is quite adequate for routine analysis of residual tetracyclines in buffalo meat below Codex MRL of 200 µg/kg.

Keywords: *RP-HPLC, Buffalo meat, Tissue residues, oxytetracycline, chlortetracycline*

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INTRODUCTION

Tetracyclines are wide spectrum bacteriostatic antimicrobials commonly prescribed in ruminants including buffaloes to combat infectious diseases like haemorrhagic septicaemia, anthrax, black quarter, leptospirosis, contagious bovine pleuropneumonia, calf scours, septicaemia, infectious mastitis (Pagel and Gautier., 2012; Chopra and Roberts *et al.,* 2001). Occurrence of antimicrobial residues is mainly accounted to non-observance of withdrawal periods and off label use. Presence of such residues in animal derived foods has attracted considerable attention owing to food safety in recent years. Of particular concern are teeth stains in children, alteration in intestinal microflora and development of resistant strains of pathogens (Cornejo et al., 2019; Molina *et al.,* 2016 and Da costa *et al.,* 2013).That's precisely why, regulatory agencies like Codex Alimentarius commission has set the maximum residue level (MRL) of 200µg/Kg (CAC/MRL 2-2015) and European Union Regulation of 100µg/Kg (EU 37/2010) to ensure food safety.

Different screening methods were in place to detect tetracyclines in different biomatrices using diverse principles like biosensor assay (Mungroo *et al.,* 2014; Pikkemaat *et al.,* 2010), microbial assay (Tumini *et al.,* 2016 and 2019; Gondova et al., 2014 and Huet *et al.,* 2008) and immunological assay (Baghani *et al.,* 2019; Wongtangprasert *et al.,*, 2014; Chafer-Pericas *et al.,* 2011) which was only qualitative /semi quantitative in nature. There were also some novel methods for detection of tetracyclines reported like Plasmon resonance (Amjadi *et al.,* 2016 and Andree *et al.,* 2010) and Quantum dots (Garcıa-Fernandez *et al.,* 2014; Li *et al.,* 2017).Many confirmatory methods using sophisticated tandem mass spectrometry with complex extraction process were already in place (Cinquina *et al.,* 2003 and Blasco *et al.,* 2009). Even then, more and more simple quantification methods using HPLC are imperative to know the levels of violation in field level. The regulatory agencies are underpinning the necessity to have analytical methods that are capable of quantifying below the MRLs.Therefore, the present study was undertaken to establish a RP-HPLC method for simultaneous quantitation of oxytetracycline and chlortetracycline in buffalo meat in compliance with Codex MRL.

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MATERIAL AND METHODS

Chemicals and reagents: The analytical standards oxytetracycline and chlortetracycline, oxalic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade methanol (MeOH) and acetonitrile (ACN) from Merck (Darmstadt, Germany) were used. Water was purified through a Milli-Q system (Millipore, Bedford, MA, USA).

Preparation of Standard and working drug solutions: Analytical grade standard of oxytetracycline and chlortetracycline hydrochloride with 99.5% purity were used for this study. Stock solution of 1mg/ml concentration of oxytetracycline and chlortetracycline were prepared in methanol. 10.8±0.1 mg each of OTC.HCL and CTC.HCL weighed and dissolved with methanol in separate 10 ml volumetric flask. A combined intermediate stock solution is then prepared for the required concentrations viz 25, 50, 100, 200, 300, 600 and 1000 ng/ml by diluting with appropriate volume of methanol. All stock and working standard solutions were stored at 4 ^{o}C in the refrigerator.

Extraction Procedure: The buffalo meat sample was extracted with Mcllvaine buffer – EDTA solution followed by solid-phase extraction clean up using Strata C18E cartridge. The eluent was pooled and evaporated using nitrogen gas at 45 ^{o}C. The extract was then reconstituted with 0.01M oxalic acid and methanol (85:15). After passing through a 0.25 µ nylon membrane filter,50 µL of this filtrate was injected into the column for HPLC analysis.

High Performance Liquid Chromatography Conditions: Tissue concentrations of oxytetracycline and chlortetracycline were assayed by reversed-phase high–performance liquid chromatography with UV detector. The UFLC system (Shimadzu Corporation, Kyoto, Japan) comprised of an LC 20AD pumps, an auto injector SIL-20AC, SPD-20AV UV-VIS detector and LabSolutions Ver. Chromatographic workstation. The separation of two analytes was achieved using a C18 reverse phase column (Phenomenex, 4.6 x 250 mm; 5^{o}m) as a stationary phase. The mobile phase consisting 0.1M oxalic acid: methanol: acetonitrile (70: 15:15, v/v) which was operated in isocratic elution mode. A flow rate of 1.5 ml/min was used, column oven temperature was kept at 40^{o}C and column pressure 240 kgf. UV detector wave length was adjusted to 360nm. Oxytetracycline and chlortetracycline were quantified from the peak areas and their respective concentrations in the calibration curves obtained from analysis of blank buffalo meat fortified with the external standards.

Calibration curve: Calibration curves were prepared by adding to blank meat samples, the corresponding volume of working solution to obtain the following concentrations of oxytetracycline and chlortetracycline: 25 µg/kg (0.125 MRL), 50 µg/kg (0.25 MRL),100 µg/kg (0.5 MRL), 200 µg/kg (1 MRL), 300 µg/kg (1.5 MRL), 600 µg/kg (3 MRL) and 1000 µg/kg (5 MRL). Residual tetracyclines were quantified in µg/ kg based on peak area measurements using external calibration method. Parameters like limit of detection (LOD), limit of quantification (LOQ) and recovery percentage were studied.

Statistical analysis: The recovery and precision data were evaluated with an in-house statistical software program making use of Snedecor and Cochran concepts.

RESULTS AND DISCUSSION

Antimicrobial residues in animal derived food has emerged as a serious concern in recent years. A simple and sensitive analytical method to quantify the veterinary drug residues in meat matrix is paramount. Therefore, an RP- HPLC method using UV detector was studied to quantify the concentrations of oxytetracycline and chlortetracycline residues in buffalo meat in compliance with Codex MRL. Many scientific literatures on quantitative analysis of oxytetracycline /chlortetracycline residues using HPLC in different biomatrices like bovine milk, meat etc using UV detector (Li, *et al.,* 2015), DAD (Ahmadi *et al.,* 2015; Zu *et al.,* 2016) and FLD detector (Pena *et al.,* 2005; Spisso *et al.,* 2007) were available. Even many works using highly sophisticated techniques like LC-MS/MS (Cammilleri *et al.,* 2019; Stolker *et al.,* 2013; Boscher *et al.,* 2010) using complex multistep extraction process with varying recovery % and LOQ were also reported.

The proposed procedure entails extraction of buffalo meat samples with EDTA-Mcllvaine buffer followed by solid-phase extraction clean-up. Instrumental analysis was performed using liquid chromatography with UV detector operated at 360 nm. Good chromatographic separation was exhibited between oxytetracycline and chlortetracycline that was achieved using 0.1M oxalic acid: methanol: acetonitrile (70: 15:15, v/v) as the mobile phase (Table 1). The whole analytical run was accomplished with in a total run time of 10 minutes.

Table 1: HPLC Conditions

System	**UHPLC**
Column	C18 4.6 X 250 mm 5µ
Injection volume	50 µl
Oven temperature	40 oC
Mobile phase	MP:0.1M oxalic acid:MeOH:ACN (70:15:15, v/v)
Flow rate	1.5 ml/min
Detector	UV λmax 360nm

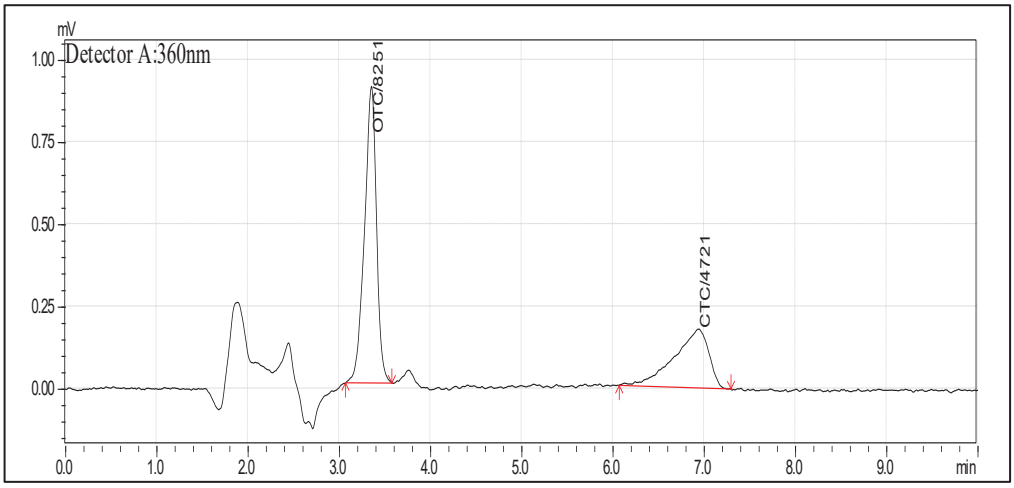

Fig. 1: Liquid Chromatogram of Oxytetracycline and Chlortetracycline using UV detector

The performance parameters demonstrated the complete adequacy of the method for detecting and quantifying the residues of oxytetracycline and chlortetracycline, in the buffalo meat in compliance with Codex MRL (Table 2). Analysis of blank muscle samples demonstrated that there were no interfering compounds at the retention times of analytes of our interest, demonstrating the selectivity of the method. The retention times for oxytetracycline and chlortetracycline were 3.3 and 6.9 minutes respectively (Fig. 1).

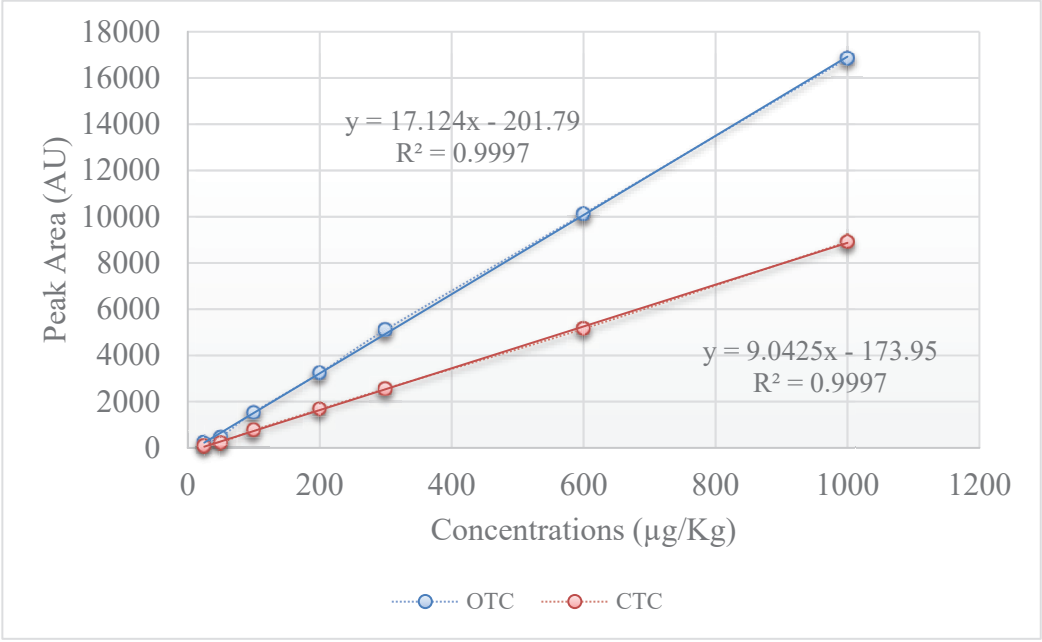

Fig. 2: External calibration curve in the concentration range of 25-1000 µg/kg

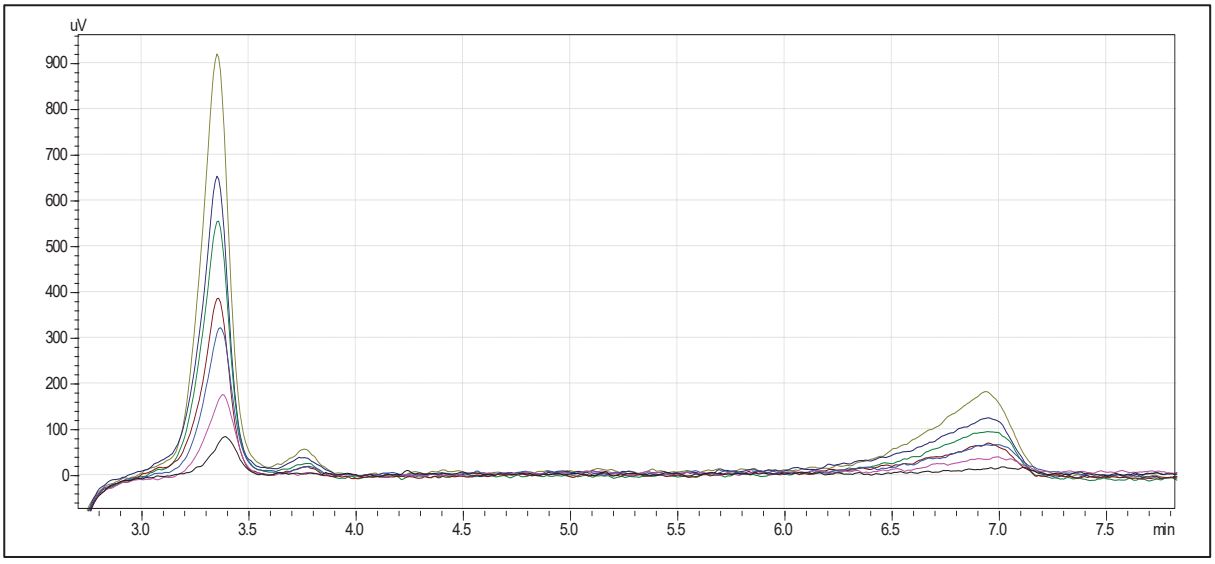

Fig. 3: Linearity overlay in the concentration range of 25-1000 µg/kg

The assay was linear from 25 to 1000 µg/ kg (Fig 2 &3). The coefficients of determination (R2) values of the calibration curves were higher than 0.99, indicating a good fit of the data to the regression line (CD 2002/657/EC (Table 2). The recoveries were investigated at three concentration levels around MRL. The percentage recovery was adequate for the method studied, they ranged from 90% to 95% (oxytetracycline) and 92% to 97% (chlortetracycline) in buffalo meat.

Table 2: Performance parameters

Oxytetracycline			Chlortetracycline		
LOD (µg/Kg)	LOQ (µg/Kg)	Recovery (%) n = 6	LOD (µg/Kg)	LOQ (µg/Kg)	Recovery (%) n = 6
12.64	38.30	90-95	12.08	36.63	92-97

The limit of quantification (LOQ) of oxytetracycline and chlortetracycline were 38 and 36 µg/kg in buffalo meat. The obtained values were remarkably lower than the Codex Alimentarius Commission MRL (200 µg/kg) established for the analyte of our interest. Our results therefore demonstrate that the proposed RP-HPLC method is adequate for the simultaneous detection and quantification of tetracyclines residue in buffalo meat below Codex MRL.

CONCLUSION

RP-HPLC method with high specificity and sensitivity has been demonstrated with good recoveries and linearity accuracy to quantitate simultaneously, oxytetracycline and chlortetracycline in buffalo meat matrix. The LOQ obtained for oxytetracycline and chlortetracycline in this assay is found to be far lower than the established codex maximum permissible limit. Therefore, this method is quite suitable to quantify the residual tetracyclines below Codex MRL (200µg/Kg) in buffalo meat samples.

COMPETING INTERESTS: No

ETHICS STATEMENT: Not applicable

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