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Solid phase extraction and detection of OTC and TC residues in milk using UHPLC-DAD

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Abstract
The present study was conducted for detection of Oxytetracycline (OTC) and Tetracycline (TC) residues in milk using Solid Phase Extraction (SPE) method and Ultra High Performance Liquid Chromatography (UHPLC) system coupled to Diode Array Detector (DAD). Raw milk samples were randomly collected from roadside milk vendors in sterile containers and then preserved until analysis. Homogenization and ultrasonication of milk samples were performed followed by ultracentrifugation, extraction and filtration by using 0.2 µm syringe filter. The Standard calibration curves of OTC and TC showed good linearity (r^2 > 0.995 and r^2> 0.991) over the range of 1.0 to 5.0 µg/g. Accuracy and recovery was in the range of 95.0-99.0% and 91.5-98.0% for OTC and TC in milk indicating that the method can be used as a validated method. The present method very precisely identifies and quantifies low level of OTC and TC in milk which is useful for monitoring purpose.

Keywords: Residues, solid phase extraction, Tetracyclines, ultra high performance liquid chromatography

1. Introduction
Oxytetracycline (OTC) and Tetracycline (TC) are two important broad spectrum antibacterial agents used widely in livestock and poultry farms. They are administered to animals not only for prevention and treatment of diseases, but also for fraudulent growth promotion. In dairy animals tetracyclines may be administered orally, parenterally or through intramammary infusion [1]. Tetracyclines may persist in the body long after their administration due to enterohepatic circulation. Improper use of these agents may result as residues in edible animal tissues and milk, which can be toxic and dangerous for human health potentially causing adverse reactions. The long-term presence of tetracycline residues may cause antibiotic resistance [2]. The wide applications of tetracyclines have led to the equally fast spread of tetracycline resistant strains of gram-positive and gram-negative bacteria, including strains belonging to pathogenic as well as non-pathogenic species. Non-pathogenic bacteria could act as a reservoir of resistance determinants, which can be disseminated by horizontal transfer into pathogens. More than thirty different tetracycline resistance genes have been characterized [3]. Superinfections may occur with many antibiotics and especially as observed in case of tetracyclines. The bacteria that colonize various sites are noted to be tetracycline resistant. Hence another antibiotic needs to be selected to combat tetracycline induced infections. The therapeutic use of antibiotics generally involves higher doses of antibiotics for relatively short period whereas prophylactic use involves moderate dose of antibiotic treatment of animals to an extended period of time.
To assure consumer's safety and high quality dairy products, raw milk must be regularly screened for the presence of drug residues. Detecting violative levels of antibiotic drug residues in milk through the use of residue screening and other qualitative tests can help prevent contaminated milk from entering the human food chain. The Codex Alimentarius Commission (2012) has established Maximum Residue limit (MRL) of 0.1µg/g for tetracycline residues in milk. Several methods including UV- spectrophotometry and chromatography have been used for the monitoring of tetracyclines in food of animal origin using complex clean up procedure [4, 5]. Jevinova *et al.* (2003) determined the residues of OTC in milk samples of cow using microbial inhibition assays [6]. Navratilova *et al.* (2009) determined the residues of tetracyclines in raw milk of cow using milk tetra sensor kit [7].

Bilandzic *et al.* (2011) reported an Immunoassay method for determination of residues of tetracyclines and few antibiotics in raw milk samples [8].

The present study is an attempt to validate a method for detection of residues of OTC and TC in milk. The present method involves simple clean up procedures including solid phase extraction and detection using Ultra High Performance Liquid Chromatography (UHPLC) coupled to Diode Array Detector (DAD) having wide scanning range.

2. Materials and Methods

2.1 Sample collection

Raw milk samples of Cow of about 100 ml were collected from roadside milk vendors. The samples were randomly collected throughout the year in sterile sample containers. The samples were labelled and transported to the laboratory in thermo-cooled containers jacketed with ice and stored till the time of analysis of the samples.

2.2 Chemical and reagents

OTC and TC standard (Sigma), HPLC grade Acetonitrile (Merck), Methanol (Merck), Water, chemicals and solvents of analytical grade were used for the study.

2.3 Apparatus

The UHPLC system (Dionex, Germany) equipped with auto-sampler, quaternary pump and DAD was used. The analytical column was reversed-phase C_{18}, 25 cm × 4.6 mm I.D., 5 m column. Refrigerated centrifuge machine, ultrasonicator (Sartorius) and homogenizer (IKA) were used for sample preparation.

2.4 Standard solutions

Stock standard solution of OTC and TC compound was prepared by dissolving 10 mg of the compound in 10 ml of methanol to obtain a final concentration of 1 mg/ml. Stock standard solutions were put in amber glass to prevent the photo-degradation. Further dilutions were made from this solution in methanol in the descending concentration of 5.0, 4.0, 3.0, 2.0 and 1.0 µg/ml respectively.

2.5 Fortification of samples

The milk samples were spiked with known concentrations of OTC and TC. Fortified samples were allowed to stand at 4°C for 1 h before analysis.

2.6 Extraction and clean up procedure

About 10 ml of the milk sample was taken in a 50 ml centrifuge tube. To it added 10 ml of 0.1 M EDTA-McIIvaine buffer (pH 4.0) followed by vigorous shaking for 5 mins. The sample was then centrifuge at 6000 rpm for 10 mins. The supernatant was collected and filtered through a Whatman filter paper No. 42. Clean up of the extract was done by using Solid Phase Extraction (SPE) method. The filtrate was loaded on a Sep-pak C_{18} cartridge preconditioned with 3 ml of methanol and 2 ml of water. The cartridge containing the sample was washed with 5 ml of water and then tetracyclines were eluted with 4.5 ml of 0.01M Methanolic oxalic acid. The extract so obtained was filtered through a syringe filter (0.2µm). Later, 20µl of the eluted sample was injected into the UHPLC system for analysis.

2.7 Chromatographic condition

A mobile phase of 0.01 M oxalic acid: Acetonitrile: Methanol (77: 18 : 5, v/v/v) was used. The flow rate was kept at 1.0 ml/min keeping mode as isocratic.

2.8 Detection and quantification

The separated TCs were detected with DAD and quantification by Chromeleon chromatographic software interfaced to a personal computer. The wavelength for the detector was set at 350 nm.

3. Results and Discussion

The present study includes an extraction step with a suitable solvent system along with clean up procedure. Since it is difficult to extract TCs from biological matrix through organic solvent so extraction technique involves McIIvaine buffer for protein precipitation. Solid phase extraction clean up step is required to isolate TCs from interference of a complex sample matrix prior to the chromatographic analysis. Separation of TC residues isolated from biological matrixes was performed on a reverse phase C_{18} column using mobile phase by isocratic elution. In multi-residue analysis of TC in food, HPLC with UV detectors were the most commonly used one because they are more readily available and convenient to use in labs [9]. In the present study, TCs were separated on the RP -C_{18} column using DAD which offers typically a wider scanning range of 350-365. It was found that the detector response factor at 350 nm was optimum for detection of wavelength for TCs. The separation and peak shapes of TCs standards were better using the present mobile phase (0.01 M oxalic acid, Acetonitrile, Methanol; 77: 18: 5 v/v/v). The flow rate was kept at 1.0 ml/min keeping mode as isocratic. Linearity was evaluated by calibration in the range of 1.0 to 5.0 µg for each compound at five points with triplicate analysis. The response of the DAD was linear and highly correlated with the amounts of TC injected, where the calculated coefficient (r^2) ranged from 0.991 to 0.995 and each TCs had own linear equation. The sensitivity of the method is usually represented by the slope of the analytical calibration curve. Accuracy and recovery was in the range of 95.0-99.0% and 91.5-98.0% for OTC and TC in milk indicating that the method can be used as a validated method. Earlier, a method was reported where recovery of TC was 70% using a reverse phase HPLC method with diode array detection for the determination of tetracycline residue in milk [10].

OTC residue was detected in 2 milk samples of Cow with concentration of 0.146 µg/g and 0.181 µg/g. Both the samples were above the MRL. Also, 3 samples of Cow milk were detected with residues of TC with level of 0.083 µg/g, 0.114 µg/g and 0.125 µg/g. Out of the 3 samples, 2 samples were found to be above the MRL. Similar finding was reported by Jevinova *et al.* (2003) where OTC residues above the MRL were detected in milk samples of cow of Slovakia [6]. Seyda and Ayhan (2010) screened 240 samples of milk for determination of antibiotic residues including OTC using TLC [11].

4. Conclusion

This study demonstrated that the present method reliably identifies and quantifies the selected TC and OTC in the reconstituted milk samples in very low range which can be applied for monitoring purpose.

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