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A Simple Multi-residue Method for Determination of Oxytetracycline, Tetracycline and Chlortetracycline in Export Buffalo Meat by HPLC-Photodiode Array Detector

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ABSTRACT

The development of a simple, rapid and sensitive method for residue monitoring of oxytetracycline (OTC), tetracycline (TC) and chlortetracycline (CTC) in buffalo meat samples is described. The principal steps involved extraction in McIlvaine buffer (pH 3.85) followed by a solid phase clean up step. In HPLC, a reversed phase C8 (RP-C8) column was used and compounds were separated at 35°C using a mobile phase of 0.01 M oxalic acid buffer (pH 1.6)/acetonitrile/methanol (77:18:5, v/v/v) at a flow rate of 0.6 mL/min. A wavelength of PDA detector was set at 355 nm. The detection limit of the method was calculated to be 0.031 μg/g and the minimum detectable quantity was found to be 0.062 μg/g. The statistical evaluation demonstrated high absolute recoveries of OTC, TC and CTC from spiked samples at three fortification levels, which were higher than 78% for all drugs. Excellent method repeatability and reproducibility was found by intra- and inter-day assay precision, yielding the coefficients of variation not more than 11.4 and 14.5% at 0.062 μg/g spike concentrations, respectively. The method was also employed for monitoring of 122 export buffalo meat samples collected from different parts of India, in which only 5 samples showed detectable concentration of OTC residues but were lower than the maximum residue limits (MRLs) set by Codex Alimentarius Commission (CAC), European Union (EU), and United States Food and Drug Administration (US-FDA). TC and CTC were absent in all samples.

Key words: oxytetracycline, tetracycline, chlortetracycline high-performance liquid chromatography, buffalo meat, residues

INTRODUCTION

In the recent year, Indian buffalo meat (*Bubalus bubalis*) taps the world markets through export trades. Meat from this species has wider acceptability due to low fat. They are raised to nearly organic farming system without any routine use of pesticides, veterinary drugs, hormonal compounds and growth promoters, and have been given sufficient withdrawal period of time before slaughter. However, in view of global competitive markets in post WTO era and imposition of Sanitary and Phyto-Sanitary (SPS) measures, there is an urgent need of a nation wide survey to know residue spectrum of buffalo meat being produced. Monitoring and strict implementation of these emerging contaminants continue to demand many positive benefits such as improved national nutritional status, major economic advantages including job creation, and improved diplomatic relationship between the member countries concerned. But this relies on testing and other forms of inspection by either exporting or importing country or both.

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Antibiotics are used in food producing animals not only to treat disease but also to maintain health and promote growth. Tetracyclines (Figure 1) are most widely used antibiotics in veterinary medicine in India due to its broad spectrum of antimicrobial activity, availability and low cost. Unauthorized use of these antibiotics, the failure to follow label directions or inappropriate withdrawal period of time before slaughtering of animals could lead to residues in food of animal origin, with potential adverse effects on human health. The overuse of OTC/TC/CTC in animal production or their residues in food system poses a potential allergic reaction in sensitized individuals, but sub-therapeutic and therapeutic levels may perturb human gut microflora by introducing resistant strains and altering the metabolic activity of the microflora, its resistant microorganisms barrier effects, and its ecological balance without any identified deleterious effects[1-5].

The Codex maximum residue limits (MRLs) set for OTC/TC/CTC (alone or combination) are 0.1 μg/g in muscle tissues. Though considerable research has been done on the determination of TCs residues in animal tissues in India, no recent scientific data are available for buffalo meat in concern. So, it is urgent to establish a monitoring program to detect TCs residues in buffalo meat.

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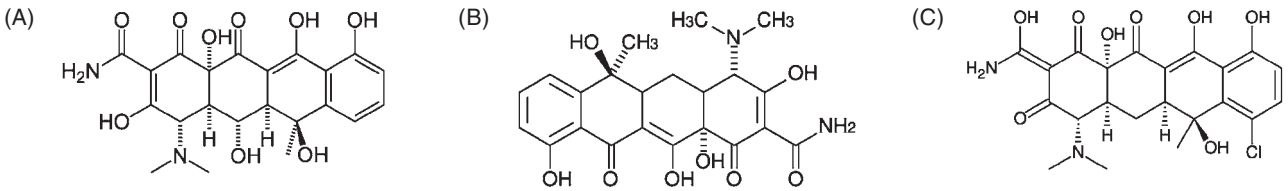

Figure 1. Chemical structure of (A) oxytetracycline (OTC), (B) tetracycline (TC) and (C) chlortetracycline (CTC).

Several chromatographic methods have been employed successfully for the monitoring of TCs in tissue samples with different detection modes such as UV-spectrophotometry, fluorescence, and mass spectrometry in the past[6-8]. All these procedures used a simple clean-up step by solid phase extraction (SPE) or matrix dispersion. The use of UV detector in residue analysis has low sensitivity, while mass spectrometry still has cost affair. In general, PDA detection is sensitive and has wide scanning range.

The objective of present study was to develop and validate an analytical methodology for specific and sensitive determination of TCs in buffalo meat by HPLC with PDA detector by following the method mentioned[8] with modification. The compounds were extracted with McIlvaine buffer (pH 3.85) and cleaned up with Oasis HLB polymeric solid phase extraction cartridge.

To enhance the precision and accuracy of the analytical method, the validation was compelled with CAC, US-FDA, Australian Pesticides and Veterinary Medicines Agencies (APVMA) and EU decision. Finally, this validated method was applied to analysis of TCs residue in buffalo meat samples.

MATERIALS AND METHODS

I. *Instrumentation*

The HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with a LC-10AT quaternary gradient pump, a Rheodyne manual loop injector 20 µL, a column oven CTO-10AS vp, a PDA detector and a software class-vp 6.12 version for data analysis. The analytical column was a Luna 5 µ C8 (RP- C8) column (4.6 × 250 mm, 5 µm particle size) from Phenomenex Co. (Torrance, CA, USA). The optimized mobile phase for desorption and separation was a mixture of 0.01 M oxalic acid/acetonitrile/methanol (77:18:5, v/v/v), and the flow rate was kept at 0.6 mL/min. The detection was performed at 355 nm with scanning range 340-360 nm.

II. *Materials*

HPLC grade acetonitrile, methanol and water were obtained from Rankem, E. Merck (India), and Qualigens Fine Chemicals. Water for HPLC was also obtained from Millipore water purification system and was filtered through 0.22 µL filter prior to use. AR/GR grade disodium hydrogen phosphate (Na_2HPO_4), citric acid and oxalic acid were purchased from Merck, Rankem and SRL (India). Oasis HLB cartridge 6 cm^3 (200 mg) was procured from Waters (USA). Pure standard of oxytetracyline (assay 96.2% in HPLC), tetracycline (assay 98.6% in HPLC) and chlortetracycline (assay 97.3% in HPLC), as their hydrochloride, were obtained from Sigma-Aldrich Pvt. Ltd., USA. Individual stock standard solutions of TCs at 1 mg/mL (free base) were prepared in methanol in an amber color volumetric flask separately and were stored at -20°C in the dark for maximum period of 2 months. A composite working standard solutions of 320, 160, 80, 40, 20, 10, 5, 2.5, 1.25, 0.625 and 0.031 µg/mL were prepared by diluting all stock solutions with methanol. As it is unstable at room temperature, so prepared daily and stored at 4°C .

For analysis, 0.01 M oxalic acid (pH 1.6), 0.1 M citric acid and 0.2 M disodium hydrogen phosphate (Na_2HPO_4) buffer were prepared in Milli-Q water and filtered through 0.22 µm cellulose filter, 0.01 M methanolic oxalic acid (pH 1.86) was prepared in methanol. McIlvaine buffer (pH 3.85) was prepared by mixing 278 mL of 0.1 M citric acid solution in 222 mL of 0.2 M Na_2HPO_4 solution and the pH was adjusted to 3.85 with extra citric acid solution. All these buffer solutions were stored at 4°C.

III. *Methods*

(I) *Sample Collections*

Buffalo meat samples comprised of 92 *Longissimus dorsi* (LD) muscle and 30 silver sides (SS) were collected from different export meat processing plants across the country, over 12 month's period. The meat was processed on the deboning table where the chilled carcasses were cut, deboned, trimmed and packed. About 100 g of tissue sample was cut from LD or SS randomly at different period of deboning operations and transferred into pre-sterilized colorless self-sealing polyethylene (PE) bags. The bags were labeled, frozen and brought to the laboratory under frozen conditions in foam box containing chiller packs. Both types of sample were stored at -20°C before analysis.

(II) *Sample Extraction*

Frozen tissue samples were thawed and finely diced with scissors after trimming off external fat and fascia.

The finely cut samples were blended in a high speed (15,000 rpm) tissue blender (York Scientific Industries Pvt. Ltd., New Delhi, India) for 2 min. A representative portion of this sample (10 g) was weighed into a polypropylene tube and homogenized with 10 mL of Milli-Q water for 1.5 min using Ultra-Turrex T25 tissues homogenizer (Janke and Kenkel, IKA, LaborTecnik, USA). Then an aliquot (0.5 g) of homogenized sample was transferred into a glass test tube, fortified with 50 µL of variable concentrations of the working standard solution, leaving the analytes in contact with meat sample for 30 min. After 3 mL of McIIvaine buffer was added, the mixture was vortexed at high speed, incubated for 5 min at room temperature and centrifuged at 3,500 rpm for 10 min in a refrigerated centrifuge (Biofuse, Heraeus, USA). The extraction was repeated by adding 2 mL of McIIvaine buffer and the supernatant was pooled. The supernatant was filtered and loaded on an Oasis HLB 6 cm3 (200 mg) polymeric cartridge previously conditioned 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 0.01 M methanolic oxalic acid (pH 1.80). One milliliter of eluent was filtered through 0.22 µm nylon filter, vortexed and centrifuged, and then 20 µL of the aliquot was injected into the HPLC system.

RESULTS AND DISCUSSION

I. *HPLC Conditions Optimization*

In multi-residue analysis of tetracycline compounds in tissue samples, HPLC with UV-detector set at 350-355 nm was most commonly used[7,9], but has low sensitivity and specificity. So, HPLC with PDA detector is getting popularity, as a wide UV spectrum (scanning range) besides its fixed wavelength maxima is covered for a particular compound. Other researchers also satisfactorily performed residue analysis of tetracyclines in milk by HPLC with diode array detector set at 365 nm wavelength without any matrix interference. In present study, the absorbance maximum was set at 355 nm with a scanning range of 340-360 nm wavelengths[8]. At wavelength of 340 nm or below, sharpening of peak has increased significantly, but simultaneously also increased matrices interference. Similarly, reverse effect was noted at wavelength of 360 nm. So, wavelength at 355 nm was fixed for residue analysis without any unwanted peak in its retention time. The mean retention time for OTC/TC/CTC was 6.9, 9.2 and 12.4 min, respectively. On the basis of 6 parallel determinations over 3 days, the coefficient of variation (CV) of the retention time was 0.38% for OTC, 0.22%for TC and 0.48% for CTC (Figure 2).

In this study an RP-C8 Phenomenex column was employed at 35°C temperature with 0.01 M oxalic acid buffer (pH 1.60)/acetonitrile/methanol (77:18:5, v/v/v) as

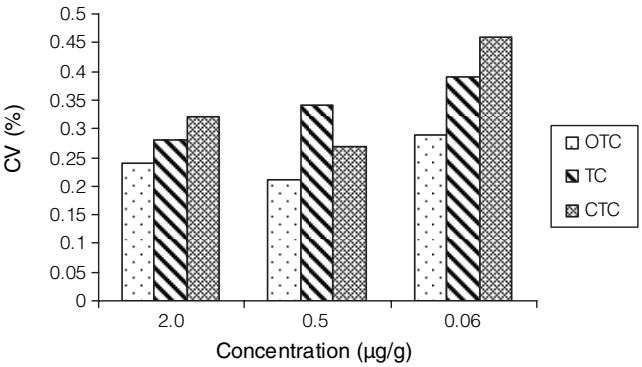

Figure 2. Coefficients of variation (CV) of retention time (RT) during HPLC analysis of tetracyclines (OTC/TC/CTC) in buffalo meat.

the mobile phase. The isocratic elution under the condition employed allows the separation of OTC, TC, and CTC with good resolution. Good separation and peak shape were obtained at relatively low flow rate of 0.6 mL/min. Same mobile phase containing oxalic acid buffer was also used for residue analyses of tetracyclines[9]. In the standardization of mobile phase, an initial attempt was made using diluted sulfuric acid (pH 2.1)/acetonitrile (85:15, v/v) by following the method of Senyuva *et al.*[10], but oxytetracycline could not be separated from the C8 column. Moreover, the pH of diluted sulfuric acid was very unstable. On the other hand, mobile phase of 0.01 M oxalic acid/acetonitrile/methanol was stable, and had the capability to separate residual components efficiently. However, a small change of flow rate and the pH of mobile phase buffer in the vicinity of pKa of oxytetracycline (pKa = 2.7) greatly affected its retention time. At high flow rate and low buffer pH, components could not be separated from the matrix interference due to reduced sensitivity and specificity of the column. So, a flow rate of 0.6 mL/min and buffer pH of 1.60 were quite suitable for the determination of TC components in the HPLC system. These are agreement with the method developed by Pena *et al.*[11], while Capolongo *et al.*[7] used mobile phase buffer of pH 2.3 and at flow rate of 0.5 and 1.0 mL/min, respectively.

II. *Sample Extraction Optimization*

Sample extraction was performed using widely acceptable McIIvaine buffer (pH 3.8) as per methodology of Kao *et al.*[12] with slight modifications. Extraction with McIIvaine buffer without Na_2-EDTA has no unwanted effect in subsequent HPLC analysis. The effect of pH of the sample matrix on the extraction efficacy of tetracyclines was evaluated using different buffer solutions with pH 2.5-5.5. As shown in Figure 3, a high extraction efficacy for all three tetracyclines was obtained at pH values of 3.5 and 4.0, and an obvious decrease was found when pH was away from this limits. The pKa values of OTC, TC and CTC are in the range of 2.7-3.5, so molecules could be

deprotonated and possess a negative charge if the solution pH is higher than pKa value of these compounds. Some researchers used polymer monolithic material to deprotonate the compounds, but we restricted the pH of extraction solution at 3.8 in order to be devoid of these unusual effects. The ion-exchange interactions of protonated drugs increase in acidic environment thereby an increase in the extraction efficacy.

For clean up, Oasis HLB 6 cm^3 (200 mg) cartridge was used. This polymeric cartridge does not contain any sianol backbone, thus enabling them as an effective sorbent which is devoid of problem of TCs interacting substances such as sianol is in silica-based cartridge. Moreover, HLB cartridge has strong affinity for TCs, through hydrophobic, hydrogen bonding and cation exchange interactions. Solvents used for conditioning/washing/elution and their volume for HLB cartridges were also evaluated. During the preliminary study of standardization, it was observed that methanol followed by water wash was more efficient in removing interfering substances than methanol and water alone. Mobile phase, 0.01 M methanolic oxalic acid and methanol have tested for efficient elution of TCs. The overall recovery efficiency of tetracyclines was as follows: 0.01 M methanolic oxalic acid > mobile phase > methanol. However, volume of the eluent was limited up to 4.5 mL as no improvement was observed in further increasing the elution volume. However, chromatogram still showed some interfering peaks, corresponding to substances which might have originated matrix effect. Similar procedures were also reported[7,9,13].

The procedures used for extraction and purification of TCs from buffalo meat sample did not require second step clean up. After first step clean up and filtration, the filtrate was clear and free of particulate matter capable of fouling the column inlet. Further, as additional precaution, a guard column was used. So, an aliquot was directly analyzed by HPLC-PAD at low pressure gradient conditions, allowing separation of parent compounds. The RP-C8 column was chosen after preliminary comparing to the RP-C18 column. The former one produced a better resolution of OTC and improved peak shape and sensitivity as expected due to its capability to bind more polar compounds at a low flow rate of 0.6 mL/min[11]. However, Kao *et al.*[12] employed a two step clean-up procedure with HLB cartridge followed by another clean-up through a COOH column. In present study, our results were in accordance with those of previously published methods[9,13]; although they used only one clean-up step, but need further study to minimize losses and to improve the method precision too.

III. *Analytical Method Validation*

To verify the absence of potential interfering substances around the retention time of TCs a number of reference blank and spiked meat samples from different regions were analyzed in order to assess the specificity of the method. No interference was observed in the region where the analytes were eluted.

Standard calibration curve of oxytetracycline in blank meat (0.031, 0.062, 0.125, 0.25, 0.5, 1.0 and 2.0 μg/g) was prepared by external standard methods. Sample and standard solutions, 20 μL of each, were individually injected to the HPLC system. Peak identification was made by comparing the retention times and spectra of samples with those of standards. The concentration of TCs in meat homogenate was determined by substituting respective peak heights in the linear regression formula. The result obtained for the method standardized for the determination of tissue residues of OTC, TC and CTC was linear in the range of 0.06-2.0 μg/g (Figures 4, 5 and 6).

The recovery and repeatability of the method were evaluated by analysis of five spiked samples with TCs at 0.031, 0.25 and 2.0 μg/g, on three different occasions (n = 15). These levels correspond to more than one-third of the maximum residue limits (MRLs, 0.1 μg/g), 2.5 times the MRL and 20 times the MRL, respectively, and are set by the CAC and EU. The estimated extraction recoveries from spiked meat samples for OTC, TC and CTC reached high values, ranging from 78.3 to 100% with relatively low coefficient of variation ranged from 0.90 to 11.0% (Table 1). The recoveries for OTC, TC and CTC

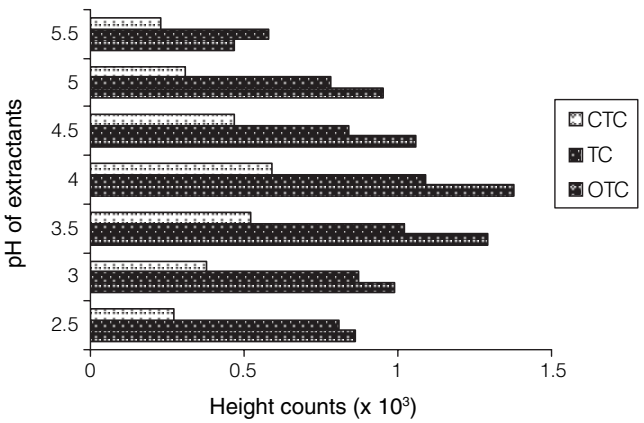

Figure 3. Effect of pH of extraction solution on recovery of tetracycline residues in spiked buffalo meat sample.

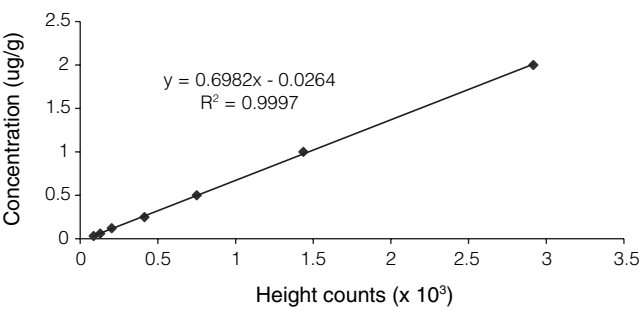

Figure 4. Linear calibration curve of the HPLC method for oxytetracycline residues in export buffalo meat.

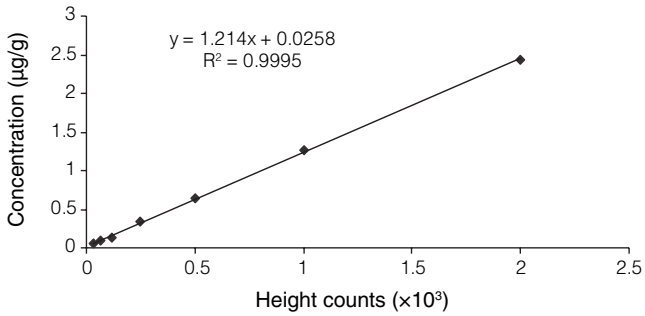

Figure 5. Linear calibration curve of the HPLC method for tetracycline residues in export buffalo meat.

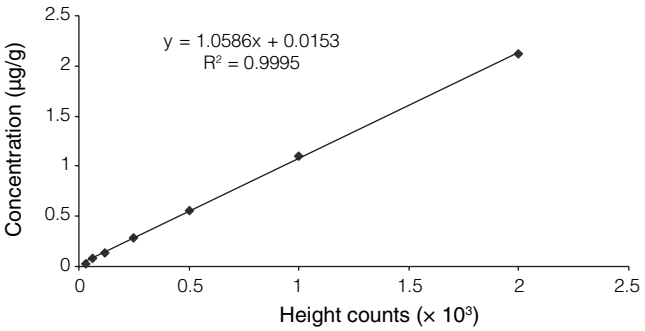

Figure 6. Linear calibration curve of the HPLC method for chlortetracycline residues in export buffalo meat.

Table 1. Recovery and precision of oxytetracycline spiked in to buffalo meat sample

Compound	Recovery[a]		Precision[b]		
	Concentration (µg/g)	Recovery (%)	Concentration (µg/g)	Inter-day assay (CV%)[c]	Intra-day assay (CV%)
OTC	2.0	98.2 (0.9)	2.0	7.2	6.2
	0.25	100.4 (5.2)	0.5	6.3	8.4
	0.031	98.6 (11.3)	0.062	14.6	11.4
TC	2.0	89.4 (2.1)	2.0	3.4	2.1
	0.25	81.7 (4.2)	0.25	5.6	4.3
	0.031	78.3 (6.4)	0.062	7.9	5.2
CTC	2.0	96.2 (5.2)	2.0	4.7	3.9
	0.25	88.6 (8.5)	0.25	5.8	4.7
	0.031	79.6 (9.2)	0.062	8.7	9.3

[a]n=5.
[b]N=15.
[c]Parenthesis indicated percentages of coefficient variation (CV%).

at 0.031 µg/g were 98.6, 78.3 and 79.6%, respectively. These results fully agreed with the recommended guidelines of CAC, European Medicines Agency (EMEA) and US-FDA for analysis of TCs in tissues of animal origin.

Inter-and intra-day assay precisions were reported in Table 1 by assessing the repeatability and reproducibility of the standardized methods. These were performed by spiking tissue samples with composite working standards at 3 levels (0.062, 0.50 and 2.0 µg/g). Data were obtained by extracting batches of 6 samples (5 spike and 1 reference blank) on three successive days for reproducibility, whereas for repeatability 18 determinants were made on each in the same day at least 6 hr apart. The blank determinants were performed to ensure that no interference from background peaks could be observed on the chromatogram. Results of these study revealed that repeatability and reproducibility were corresponding to the validation methods of CAC and US-FDA with coefficients of variation of 6.21 to 11.45% and 7.2 to 14.56%; 2.1 to 5.2% and 3.4 to 7.89%; and 3.9 to 9.3% and 4.7 to 8.7%, for OTC, TC and CTC, respectively.

Limit of detection (LOD) and limit of quantification (LOQ) of the assay method were determined as per proposed guidelines of CAC, EU, US-FDA, and APVMA. Results showed that the detection limit of the tested drugs (TCs) was 0.031 µg/g. For measurement, consideration was given only when the first condition was satisfied for ascertaining the presence of target compounds i.e. TCs. So, the LOD was calculated accordingly, as the amount corresponding to the mean value plus three times the standard deviation for the blank sample was equivalent to 0.031 µg/g with a signal/noise ratio of 3 (S/N = 3).

The LOQ calculated on spiked tissue sample was 0.062 µg/g. These results indicated that at this concentration accuracy and precision were very similar to those explained in Table 1. So, LOQ was started from this concentration. In this case of measurement, first background noise was estimated based on the peak to peak baseline near the analyte peak. Then the peak height to averaged background noise ratio was measured. The LOQ was calculated on the basis of minimal accepted value of S/N 10.

As the EU, CAC and US-FDA have set the MRL of TCs in bovine tissue sample of 0.10 µg/g, the method

Table 2. Distribution of tetracycline residues (OTC, TC[a] and CTC[a]) in survey buffalo meat samples

No. of sample	Sample type	[b]Mean (μg/g)	Median (μg/g)	Samples contain residues (%)
92	LD[c]	0.083	0.052-0.10	6.52
30	SS[d]	0.082	0.060-0.108	13.3
122	Overall	0.082	0.052-0.108	7.73

[a]Not detected in any sample.
[b]All samples showed residues of oxytetracycline below the Codex MRL (0.1 μg/g).
[c]LD: *Longissimus dorsi*.
[d]SS: silver side.

is more than adequate to meet the needs of regulatory enforcement because the detection limits and LOQ for OTC were 0.031 μg/g and 0.062 μg/g, respectively. The method was also rapid enough to be used for direct screening of tissue samples for TCs.

IV. *Application to Real Samples*

A total of 122 tissue samples comprising of *Longissimus dorsi* (92 samples) and silver sides (30 samples) muscle were collected from export oriented slaughterhouses across the country, and were analyzed in the laboratory for the presence of TCs residue in meat. The results of analytical data of tissue samples are shown in Table 2.

The residual concentrations of OTC in *Longissimus dorsi* (LD) muscles ranged from 0.052 to 0.10 μg/g with statistical mean of 0.083 μg/g. None of the sample showed residues above the MRLs of CAC or EU although 6 (6.52%) tissue samples were detected positive for OTC.

On the other hand, mean residual concentration of OTCs in silver sides (SS) muscle was 0.082 μg/g and the range covered from 0.060 to 0.108 μg/g. In fact, both types of muscle had nearly equal concentration of OTC residues though ranges were numerically variable. Likewise, in LD samples residue concentration was merely lower than statutory limits recommended by Codex committee. However, incidences of residue in SS muscles were more than LD muscles (13.3% vs. 6.52%). The presence of this component in tissue samples might be due to slaughtering of animals without giving adequate withdrawal period of time. In a survey of market buffalo meat, Kumar[14] also reported the presence of OTC residues up to 22.33% samples, but none of them had residues concentration above MRL. In contrast to Indian buffalo meat, residues of OTC also reported in Canada (1% above MRL in pig), Korea (6% above MRL in beef), USA[15,16]. No TC or CTC residue was observed in any of the analytical samples.

CONCLUSIONS

A simple, rapid and sensitive HPLC method for the monitoring of tissue residues of TCs in buffalo meat samples was modified and validated. The clean-up procedure is a critical step as meat extract is the most complex matrix. However, clean-up using polymeric Oasis cartridges could sufficiently eliminate interfering substances at the respective peak height of the drugs. The levels of TCs compounds were investigated in 122 typical meat samples. The results show that only OTC concentrations were at 0.08 μg/g levels in few samples. Although these levels were not thought able to induce adverse effects, from a food safety perspective, low level occurrence of OTC should nevertheless be of concern.

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