Short Communication

High-performance liquid chromatography method validation for determination of tetracycline residues in poultry meat

Abstract

Background: In this study, a method for determination of tetracycline (TC) residues in poultry with the help of high-performance liquid chromatography technique was validated. **Materials and Methods:** The principle step involved in ultrasonic-assisted extraction of TCs from poultry samples by 2 ml of 20% trichloroacetic acid and phosphate buffer (pH 4), which gave a clearer supernatant and high recovery, followed by centrifugation and purification by using 0.22 μm filter paper. **Results:** Validity study of the method revealed that all obtained calibration curves showed good linearity (r^2 > 0.999) over the range of 40-4500 ng. Sensitivity was found to be 1.54 and 1.80 ng for oxytetracycline (OTC) and TC. Accuracy was in the range of 87.94-96.20% and 72.40-79.84% for meat. Precision was lower than 10% in all cases indicating that the method can be used as a validated method. Limit of detection was found to be 4.8 and 5.10 ng for OTC and TC, respectively. The corresponding values of limit of quantitation were 11 and 12 ng. **Conclusion:** The method reliably identifies and quantifies the selected TC and OTC in the reconstituted poultry meat in the low and sub-nanogram range and can be applied in any laboratory.

Key words:
Extraction, high-performance liquid chromatography, poultry, residues, separation, tetracycline

Introduction

Tetracyclines (TCs) are an important group of antibiotics used in livestock and poultry production. TCs are given to animals destined for human consumption not only to prevent and treat certain diseases, but also to fraudulently promote growth.[1] Improper use of TCs may result as residues in edible animal tissues, which can be toxic and dangerous for human health and potentially cause allergic reactions. The long-term presence of TCs residues may generate the evolution of micro-organisms provoking resistance to antibiotics.[2] For human health, the maximum residue level values of TCs residues in animal products have been established by Codex Alimentarius Commission is 200 ng/g in muscle, 600 ng/g in liver, 1200 ng/g in fat and kidney (The Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives, 2004). Therefore, the study was planned to validate this method, which is needed to detect the level of TCs in animal products.

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Sample preparation includes an extraction step with a suitable solvent system along with clean up procedure. It is difficult to extract the TCs from biological materials through organic solvent,[3] due to less log P value. So extraction technique involves acidic phosphate buffer at pH 4.0 by protein precipitation with 20% trichloroacetic acid (TCA) as well as with perchloroacetic acid. But TCA gives good recovery compare to perchloroacetic acid. Though an additional extract clean up to isolate TCs from interference of a complex sample matrix using solid phase extract and/or liquid-liquid extraction technique is required prior to the chromatographic analysis. Separation of TC residues isolated from biological matrixes was performed on a variety of high-performance liquid chromatography (HPLC) columns using different mobile phases by isocratic and gradient elution. Reversed-phase HPLC is the preferred mode although ion-exchange can also been used. The most widely used reversed-

Vikas Gupta, Sushma Ahlawat, Bhavesh Patel, Om Shankar, Kuldeep Kumar, Sanjeev K. Shukla, Shubhra Shukla, Navneet Kaur, Manisha Dubey, Lakshya Veer Singh

Department of Biochemistry and Bioprocess Technology, Sam Higginbottom Institute of Agriculture, Technology & Science, Allahabad, Uttar Pradesh, India

Address for correspondence:
Dr. Vikas Gupta,
Sam Higginbottom Institute of Agriculture, Technology & Science,
Allahabad - 211 007, Uttar Pradesh, India.
E-mail: vikas.biochem008@gmail.com

phase column type was C18 column. A great variety of mobile phases has been used such as phosphoric acid or phosphate buffers at acid pH, citrate buffers and phosphate buffer[4] and organic modifiers include acetonitrile,[5] methanol[6] and acetonitrile-methanol.[7] The detection of TCs residue is, generally done by a suitable detector like as ultraviolet (UV) and florescence detectors. Therefore, the present work is an attempt to develop an accurate, precise and sensitive HPLC-diode array detector (DAD) method.

Materials and Methods

Standard solutions

Stock standard solution of each 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 and stored at –20°C. The solution was stable for at least 4 weeks. Stock solutions were diluted with methanol water (50:50) to give a series of working standard solutions that were prepared weekly.

Fortification of samples

Fortified by 0.9% saline solution, chicken samples were spiked with TCs standard mixture at two levels of 100 ng and 200 ng of each compound per gram meat. Fortified samples were allowed to stand at 4°C for 1 h before analysis.

Extraction procedures

Take 5 g sample and put it in 50 ml capacity polypropylene tube and then add saline solution and acetonitrile, homogenate with homogenizer in ice bath using TCA as an extracting solvent then centrifuge with cooling centrifuge.

Clean up techniques

The cleanup of the aqueous based extracts was performed on 0.22 μm Millipore filter.

Apparatus

The HPLC system of a HP 1100 chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with an auto-sampler set at 150l, quaternary pump and a DAD was used. The analytical column was reversed-phase (Phenomenex 100 C18, 25 cm × 4.6 mm I.D., 5 m, Germany). The oven temperature was set at 20°C. Refrigerator centrifuge, ultrasonic bath (Buhler, Hechingen, Germany) and homogenizer (Mechanika Precyzyjna, Model type ST-2) were used for sample preparation.

Chromatographic separation of TCs

Various mobile phases were evaluated for separating TCs on a Phenomenex 100 C18 column coupled with a guard column. The mobile phases tested were as follows:

I. The mixture of acetonitrile/50 mM potassium dihydrogen phosphate (40:60, v/v) was used as an isocratic elution at flow rate of 1 ml/min.
II. The mixture of methanol/acetonitrile/50 mM potassium dihydrogen phosphate (10:30:60, v/v/v) was used as an isocratic elution at flow rate of 1 ml.
III. The gradient elution using methanol (a) acetonitrile (b) 50 mM phosphate buffer (c) was applied starting, by volume, with 5:25:70 (a:b:c) which remained isocratic for 7 min, changing to 10:25:65 in 5 min, then to 17.5:22.5:60 after 4 min and going back to the initial conditions in 3 min.
IV. The gradient elution using methanol (a) acetonitrile (b) and 50 mM phosphate buffer (c) was applied starting, by volume, with 0:8:92 (a:b:c) which changed to 0:18:82 in 2 min and to 5:20:75 in 0.1 min which remained isocratic for 4.9 min, then changed to 10:25:65 in 3 min, then to 15:20:65 in 1 min and to 15:25:60 in 3 min, which remained isocratic for 2 min and going back to the initial conditions in 3 min.

Detection and quantitation

The separated TCs were detected with DAD and the quantitation was integrated by Chemstation chromatographic software interfaced to a personal computer.

Results and Discussion

Selection of the optimum detection wavelength

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 particularly in the developing countries. DAD is getting popularity, TC is typically read at the range of 350-365 nm in an acidic solution.[3] In the present study, TCs standard solution were separated on the Nucleosil 100 C18 column and two wavelengths, i.e., 351 and 365 nm, were tested to monitor the separated TCs by measuring the response factor of the DAD. It was found that the detector response factor at 365 nm was 52.97 mAU for TC. The corresponding values obtained at 351 nm were 136.14 and hence, 351 nm selected as optimum detection wavelength for TCs determination and used throughout this study. Therefore, selection of mobile phase to separate TCs on the Phenomenex 100 C18 column is needed. In the present work, both isocratic and gradient elution were evaluated for their efficiency to separate TCs. The separation and peak shapes of TCs standards were better than those obtained with the other tested mobile phases. As well as, good resolution (Rs) between the examined chromatogram peaks was assured by the obtained values of Rs with regard to the previous eluted peak, using the equation: Rs = 2 (retention times [tR2 – tR1])/(wh1 + wh2), were 2.7, for oxytetracycline (OTC)-TC. Chromatographic parameters, i.e., tR, capacity factor (*k*) and peak symmetry of the separated TCs peaks were calculated [Table 1 and Figure 1].

Optimizing recovery

In order to obtain optimal extraction efficiency, various widely acceptable extraction solutions like McIlvaine buffer

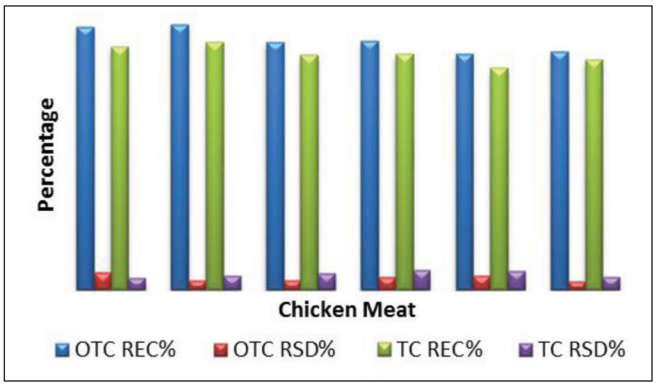

Figure 1: Clean up step recovery of tetracyclines

Table 1: Clean up step recovery of TCs from spiked extracts

Extracts	Chicken meat				
	TCs added (ng)	OTC		TC	
		REC %	RSD %	REC %	RSD %
Phosphate buffer	500	97.12	6.45	90	4.6
	1000	98.21	3.5	91.47	5.2
McIlvaine buffer	500	91.5	3.66	86.7	6.21
	1000	92	4.85	87.12	7.32
Organic	500	87.5	5.4	82	7.12
	1000	88	3.33	85	4.85

REC – Recovery; RSD – Relative standard deviation; TC – Tetracycline; OTC – Oxytetracycline

Table 2: Linearity, sensitivity, LOD and LOQ of the suggested method

Antibiotics	Slope	Intercept	r^2	LOD (ng/g)	LOQ (ng/g)
Oxytetracycline	1.44	−25.55	0.9991	4.8	11
Tetracycline	1.9	−82.6	0.9997	5.10	12

LOD – Limit of detection; LOQ – Limit of quantitation

(procedure B) as aqueous based extraction and acetonitrile (procedure C) as organic based extraction were tested to extract TCs from the samples fortified with a mixture of the two TCs at level of 100 and 200 ng/g meat.

Linearity and sensitivity

The study was extended to assay the validity of the suggested method. Linearity was evaluated by calibration in the range of 40-4500 ng for each compound at five points with triplicate analysis. The obtained results are given in Table 2. It is clear that the response of the DAD was linear and highly correlated with the amounts of TC injected, where the calculated coefficient (r^2) ranged from 0.9991 to 0.9998 and each TCs had own linear equation. The sensitivity (the change in analytical signal units per nanogram analyte) of the method is usually represented by the slope of the analytical calibration curve.[8] The sensitivity of the suggested method was found to be 1.54 and 1.80 ng/g for OTC and TC.

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

The LOD is considered to be the quantity yielding a detector response approximately equal to thrice the background noise.[3] The LOQ is the lowest amount that can be analyzed within acceptable precision and accuracy at signal to noise ratio of 10.[9] Accuracy was in the range of 87.94-96.20 and 72.40-79.84. Both LOD and LOQ were measured and the obtained data were given in Table 2. It could be observed that LOD of the suggested method was 4.8 and 5.10 ng for OTC, TC. The corresponding values of LOQ were 11 and 12 ng.

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