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## Research Article

### A RAPID METHOD TO DETECT ANTIBIOTICS RESIDUES IN HEN EGGS BY USING RP- HPLC

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#### Abstract

The objective of this study is to quantify the antibiotics (Oxytetracycline & Enrofloxacin) residue levels in domestic and layers by RP-HPLC. This study was carried out on one hundred randomly collected domestic and layers hen egg samples (20 of each) to evaluate the antibiotic (oxytetracycline and enrofloxacin) residues level qualitatively by high performance liquid chromatography (HPLC). The separation was carried out using mobile phase composed of 0.1% formic acid: Acetonitrile (50:50, v/v) using C<sub>18</sub> (Hypersil ODS-BPS, 250 x 4.6mm; 5μ) at a flow rate of 1ml/min. The residues were quantified at detection wave length 350 nm. The obtained results revealed a detectable level of oxytetracycline and enrofloxacin residues which confirm widespread misuses of antibiotics in farms and lack of application of recommended withdrawal times. The boiling effect of broiler hen egg results shows the reduction in concentration of antibiotic residues. These findings recommended that restricted measures and harder regulations must be applied to prevent the misuse residues prior to marketing.

**Keywords:** Antibiotic, oxytetracycline, enrofloxacin, Maximum residue limit, HPLC.



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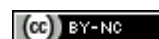
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## INTRODUCTION

Antibiotics usage has facilitated the efficient production of poultry, allowing the consumer to purchase, at a reasonable cost, high quality meat and eggs. Although these uses benefit all involved, unfortunately, the edible poultry tissues had harmful concentrations of drug residues [1-6]. These were used extensively in poultry industry for the treatment of several diseases, as well as to improve feed efficiency and promote growth. This wide spread use of antibiotic may cause residuals in foodstuffs, as well as the induction of allergic reactions in humans. Oxytetracycline is a natural tetracycline compound that is derived from the fungus, *Streptomyces rimosus* [7-11]. It is a broad-spectrum antibiotic with bacteriostatic activity; it is poorly metabolized in target animals and excreted practically in its parent form, due to its high water solubility. Enrofloxacin is a second generation fluoroquinolone with bactericidal activity. After oral application, is well absorbed and distributed at tissue level, metabolized in the liver, generating its major active metabolite, ciprofloxacin.

Many chromatographic methods are also used with diode array and fluorescence detection or liquid chromatography mass spectrometry (LC-MS) for the determination of Oxytetracycline & Enrofloxacin in hen eggs [12-15]. However; no current public reports describe a method for the simultaneous determination of OXY and ENR in hen eggs by RP-HPLC [16-18]. Therefore, a specific and sensitive method for the simultaneous determination and confirmation of OXY and ENR residues in hen eggs needed. The objective of this study is to quantify the antibiotics (Oxytetracycline & Enrofloxacin) residue levels in domestic and layers (Mature female chickens kept for egg production; also called laying hens) hen eggs by RP-HPLC.

## MATERIALS & METHODS

### INSTRUMENTATION

Method development and validation was performed on Stainless steel Hypersil ODS-BPS, C18 (250 x 4.6 mm, 3.5 $\mu$ ) column. The HPLC system includes HPLC pump (Agilent 1200 Infinity series) and UV detector (Agilent 1200 Infinity series). The software used was Ezichrome Elite Compact.

### CHEMICALS & MATERIALS

Oxytetracycline & Enrofloxacin (Hetero labs, Hyderabad), Formic acid AR (Qualigens), HPLC grade acetonitrile and grade water (Merck India), Hydrochloric acid (Merck India), (Merck India). Domestic and layers hen eggs (20 of each) were purchased local hatchery.

### Chromatographic conditions

The mobile phase composed of 0.1% formic acid: Acetonitrile (50:50 v/v) using C18 (Hypersil ODS-BPS; 250 x 4.6mm; 3.5 $\mu$ ) at a flow rate of 1ml/min. The residues were quantified at detection wavelength 350 nm. The total analysis run time was 10 min at a flow rate of 1.0 mL/min. The temperature of the column oven set to ambient temperature. Sample volume for injection into HPLC was adjusted to 20  $\mu$ L for better chromatography. The retention time of OXY and ENR were eluted at 2.7 $\pm$ 0.02 min and 5.2 $\pm$ 0.02, respectively.

### STANDARD SOLUTIONS

The individual standard solutions of the pure chemicals were prepared by dissolving accurately 10 mg OXY and ENR in a 10 mL volumetric flask using acetonitrile. Intermediate and calibration solutions were prepared by with mobile phase water prior to use. The solutions were stored in refrigerator for further use.

### SAMPLE EXTRACTION

The boiled hen eggs samples were finely sliced (Boiled at 100  $^{\circ}$ C up to 20min in double distilled water) and homogenized for 2 min. To each 5g of sample (Boiled and Un-boiled hen eggs) 20 ml of 1M HCl: acetonitrile (1:1) was added and the mixture sonicated followed by vortexed for 10 min. The tube was centrifuged for 5 min at 4000 rpm and supernatant was filtered through a 0.45  $\mu$ m nylon filter. After filtering supernatant was evaporated at 120  $^{\circ}$ C. The residue was dissolved in mobile phase and 20  $\mu$ L of solution injected was into HPLC for analysis.

### METHOD VALIDATION

The method was validated according to the Decision 2002/657/EC under Council Directive 96/23/EC and the analytical parameters were specificity, linearity, LOD, LOQ, accuracy and precision.

## SPECIFICITY

The specificity of the proposed HPLC method was performed by analyzing ten eggs samples from different sources to evaluate possible endogenous interferences in samples. The sample preparation & clean-up and chromatographic conditions were optimized to guarantee that no interferences incurred around the retention time of the tested residues.

## LINEARITY

Linearity of the method was established through a calibration curve by triplicate analysis of the five concentration levels in the matrix. The spiked concentrations were 0.05, 0.1, 0.2, 0.4 and 0.6 µg/ml for Oxy and ENR. The responses were measured as peak areas.

## LOD AND LOQ

The LOD was calculated by the comparison of the threefold variation of signal to noise ratio (S/N=3:1) obtained from analysis extract of blank eggs samples, while the lowest level that gave reasonable accuracy and precision was designated to be LOQ.

## ACCURACY AND PRECISION

The accuracy of the method was expressed as the average recoveries of spiked analytes in eggs samples. The average recoveries were measured in eggs samples known to be spiked at three different levels with triplicates over a period of five days. The recoveries were calculated by comparing peak areas of measured and spiked concentrations. The precision was defined by the relative standard deviation (RSD) and evaluated in terms of intra-day precision and inter-day precision. Intra-day precision was conducted at three fortification levels by the analysis of spiked samples in five replicates on the same day. Inter-day precision was determined for the same fortification levels of spiked samples in five replicates on five consecutive days.

## RESULTS

The proposed high-performance liquid chromatographic method has to been evaluated over the accuracy, precision and linearity and proved to be more convenient and effective for the quality control and identity of ENR and OXY in hen eggs.

The developed method was found to be simple, precise, accurate, specific, selective and linear over the concentration range of 0.05-0.9µg/mL with a correlation coefficient better than 0.998 and 0.996 for ENR and OXY respectively. Moreover, the lower solvent consumption (1mL/min) along with the short analytical run time of 10.0 minutes leads to an environmental friendly chromatographic procedure that allows the analysis of a large number of samples in a short period of time. The precision values were within 2% RSD and the % recovery was 85 and 95% for OXY and ENR respectively. The LOD & LOQ values were found to be 0.05 µg/mL and 0.02 µg/mL for OXY and 0.05 µg/mL and 0.001 µg/mL for ENR respectively. The results were depicted in Tab.1 and Fig.1&2.

**Tab.1: Precision and accuracy data**

Spiked Drug concentration (µg/mL)	Intraday (n=6)						Interday (n=30)					
	Mean±SD		Precision (%RSD)		%Accuracy		Mean±SD		Precision (%RSD)		%Accuracy	
	OXY	ENR	OXY	ENR	OXY	ENR	OXY	ENR	OXY	ENR	OXY	ENR
0.10	0.101	0.101	1.23	1.45	95.63	96.22	0.102	0.104	1.06	1.17	96.53	94.21
0.20	0.201	0.201	0.98	1.86	96.21	94.21	0.200	0.203	0.84	1.05	97.01	95.68

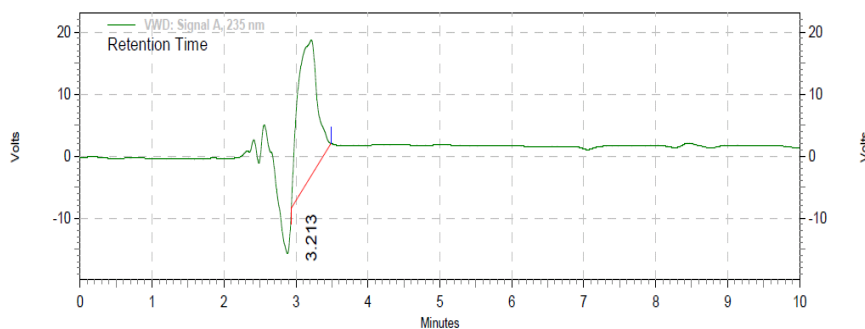


Fig.1: Chromatogram blank sample of hen egg sample

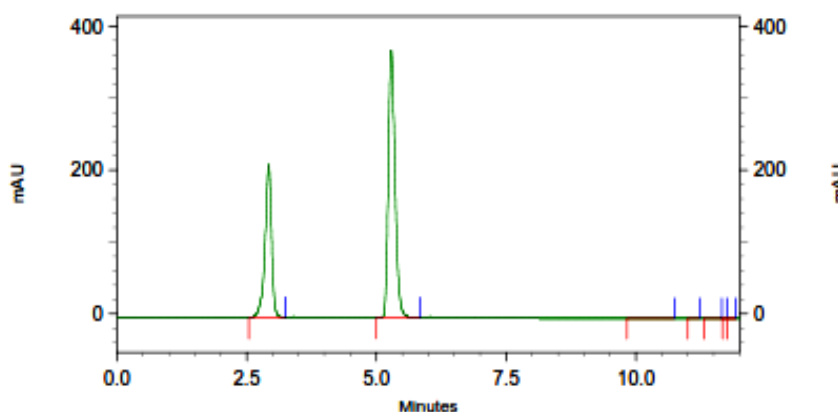


Fig.2: Chromatogram OXY and ENR residues in hen egg sample.

The positive samples resulted from HPLC assay for quantification of oxytetracycline and enrofloxacin residues. Twenty egg samples were collected from each case and analyzed. The mean residual level is 0.635µg/g for enrofloxacin and 0.526µg/g for oxytetracycline in layers hen egg samples. The residual level is 0.089µg/g for enrofloxacin and 0.062µg/g for oxytetracycline in domestic egg samples. The results were depicted in Tab.2 and Fig.3&4.

Tab.2: Antibiotic residue levels in raw and layers hen eggs.

Antibiotic	Sample number	Mean residue level (µg/g)								MRLs (µg/g) according to EC (2010)
		Un-boiled domestic hen egg (µg/g)		Un-boiled layers hen egg (µg/g)		Boiled domestic hen egg (µg/g)		Boiled layers hen egg (µg/g)		
		Mean±SD	% CV	Mean	% CV	Mean±SD	% CV	Mean±SD	% CV	
Enrofloxacin	20	0.089±0.01	1.22	0.635±0.02	1.03	0.064±0.01	1.04	0.178±0.02	0.95	0.1
Oxytetracycline	20	0.062±0.02	0.65	0.526±0.05	0.95	0.055±0.02	1.19	0.159±0.04	1.22	

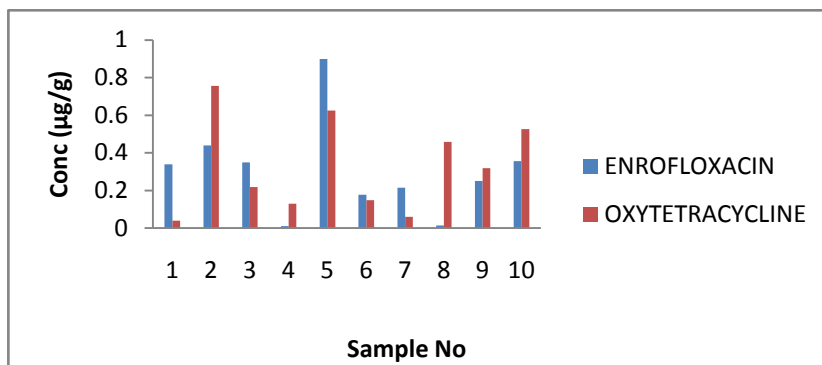


Fig.3: Detectable residue levels (µg/g) of Enrofloxacin and Oxyteracycline in layers eggs.

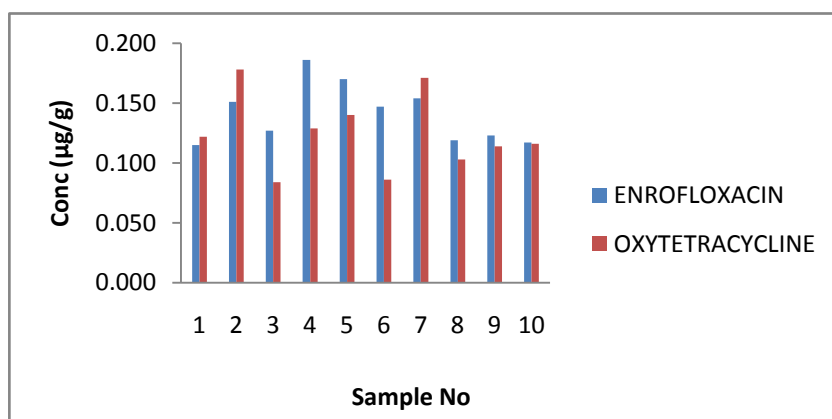


Fig.4: Detectable residue levels (µg/g) of Enrofloxacin and Oxyteracycline in domestic eggs.

In this study evaluated the effect of boiling on the concentration of antibiotic residues in layers hen eggs diminishes the concentration of antibiotic residues of oxytetracycline up to 0.178µg/g and for enrofloxacin 0.152µg/g. The results were depicted in Fig.5.

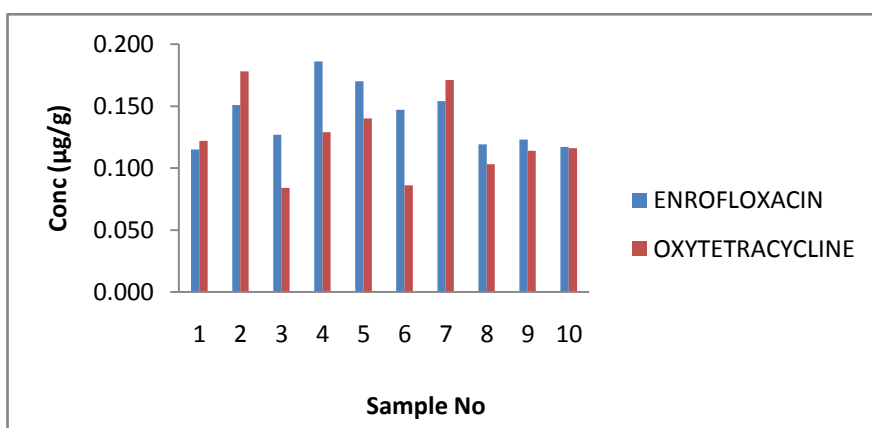


Fig.5: Detectable residue levels (µg/g) of Enrofloxacin and Oxyteracycline in boiled layers eggs

**DISCUSSION & CONCLUSIONS**

European Union commission recommended the MRLs of oxytetracycline and enrofloxacin in the edible tissues of food producing animals were 0.1µg/g (EC, 2010). While enrofloxacin and

oxytetracycline broiler egg samples were exceed MRLs, this may attributed to lack of implementation of recommended withdrawal time and the unpaid attention to the withdrawal period of eggs. In addition human exposure to animal products containing significant level of antibiotic residues

may cause disorder of intestinal flora. This study stresses on the need for stricter regulation for the use of drugs in the poultry industry as well as the inspection of broilers for residues prior to marketing.

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### AUTHOR CONTRIBUTION

All authors Contributed Equally.

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