Full Length Article

VALIDATION OF SIMPLE ISOCRATIC HPLC ASSAY FOR THE DETERMINATION OF CHLORTETRACYCLINE CONCENTRATION IN PLASMA OF CHICKEN

M. Nivetha[1], S. Ramesh*[2], L. Kalaiselvi[3], R. Gokul[4] and T. Ramasamy[5]

Department of Veterinary Pharmacology and Toxicology
Madras Veterinary College
Tamil Nadu Veterinary and Animal Sciences University
Chennai – 600 007

ABSTRACT

Chlortetracycline (CTC) is a broad-spectrum antibiotic belonging to the tetracycline group of drugs. It is commonly used in the poultry industry for the treatment of bacterial infections and is also used as a growth promoter. This study was aimed at validating the sensitive HPLC method for the assay of CTC in chicken plasma. The method consisted of isocratic elution with separation using a C_{18} column. The mobile phase used in the study was aqueous oxalic acid (0.03M), acetonitrile and methanol in the ratio of 60:30:10. The HPLC conditions included a flow rate of 1ml/min with UV detection at 375nm. CTC in chicken plasma was extracted with Mcllvaine buffer followed by dilution with water in the ratio of 1:1 and filtered using 0.22µ filters. The peak of chlortetracycline was noticed at 5-6 minis. The method was linear from 0.05µg/ml to 10 µg/ml. The recovery percentage from plasma was recorded as 110%. Thus, from this study it is inferred that this method is optimal for the assay of CTC in plasma for pharmacokinetic studies.

Keywords: Chlortetracycline, HPLC, chicken plasma, pharmacokinetics

Received : 23.05.2024 Revised : 27.09.2024 Accepted : 08.10.2024

INTRODUCTION

The poultry industry is well-developed worldwide. It is the largest supplier of animal protein. The poultry industry in India also significantly contributes to the country's economy. One commonly used approach to enhance growth and prevent infections in poultry production is by treating with Antimicrobial Growth Promoters (AGP) (Van Boecker *et al*., 2015). Chlortetracycline is a commonly used antibiotic as AGP in the poultry industry. Chlortetracycline (CTC) was first isolated from *Streptomyces aureofaciens* in 1947. In the poultry industry it is used for the control of infectious synovitis (*Mycoplasma synoviae*), chronic respiratory disease (CRD) and air sac infections (*Mycoplasma*

[1]M.V.Sc Student
[2]Professor and Head, Corresponding author Email id: ramesh.s@tanuvas.ac.in
[3]Assistant Professor
[4]M.V.Sc Student
[5]Assistant Professor

gallisepticum, Escherichia coli),in chickens and turkeys it is used for treating complicating bacterial organism associated with the blue comb (transmissible enteritis; coronaviral enteritis), and treatment of fowl cholera caused by *Pasteurella multocida*. It is administered *en masse* in either feed or water causing residues to be deposited in the eggs and meat of chicken (Smith *et al*., 2007). However, studies on the pharmacokinetics of CTC after administration through unconventional routes such as mixing with drinking water or mixing with feed are limited. Considering their frequent use, their pharmacokinetics and thereby the dosage regimen has to be standardized in poultry for administration through these routes. For carrying out such studies, a sensitive assay method for the detection of CTC in plasma of chicken has to be standardized. In this study, an HPLC method for the assay of CTC in chicken plasma is described.

MATERIALS AND METHODS

Drugs and chemicals

Pure chlortetracycline standard (91.7%) was purchased from M/s Sigma Aldrich Private Limited, Bangalore. HPLC grade acetonitrile and methanol purchased from M/s Merck Specialities Private Limited, Mumbai was used. Oxalic acid and all other chemicals used in the study were of analytical grade.

Separation of plasma

Blood samples collected from drug free birds were centrifuged at 1500x g for 10 min for getting plasma. The plasma samples were stored at -20°C until assayed.

Extraction procedure

Chicken plasma samples were spiked with known concentrations of CTC and were subjected to liquid-liquid extraction. To 200 µl of CTC-spiked chicken plasma, 300 µl of McIlvaine buffer (1:1.5) was added and vortexed for 30 sec, followed by centrifugation at 1800 × g for 10 min. To the supernatant, water was mixed in 1:1 ratio, vortexed and filtered. 20 µl of filtrate was injected into HPLC.

HPLC instrumentation and chromatographic conditions

A high-performance liquid chromatography (HPLC; Waters, USA) system was used for the analysis of plasma for CTC. The HPLC system consisted of a pump (Model, 515), rheodyne manual injector with a 20 µL loop, UV-vis detector (Model 2489), temperature control module and Empower software for data analysis.

The chromatographic separation was performed on a reverse phase C18 column (Thermofisher Scientific *Syncronis*, 250 X 4.6 mm, 5 µm particle size) with isocratic elution. The column oven was maintained at a temperature of 32^{O}C. The mobile phase was sent at a flow of 1 ml per minute and the volume of sample injected was 20 µl.

Validation of the analytical method

The analytical method was validated in terms of sensitivity, specificity, linearity, recovery and precision.

Sensitivity of the assay

The sensitivity of the assay was expressed in terms of the Limit of detection and Limit of Quantification. The LOD and LOQ of the method used were calculated as per the following formulas (ICH guideline, 2022).

LOD = (3.3 x intercept)/ slope;

LOQ = (10 x intercept)/slope.

Specificity of the assay

The specificity was evaluated to ensure that no interference from the components present in the matrix/solvent. It was studied by comparing a chromatogram of blank, CTC in solvent, chicken blank plasma and chicken plasma spiked with CTC.

Linearity

Chicken plasma was spiked with CTC at six concentration levels (0.05 to 10 $\mu g.ml^{-1}$). The samples were then extracted and analysed. The calibration curve was created by plotting the peak area in the Y axis against the concentration of the drug in the X axis. The correlation coefficient (r^2) value was calculated.

Recovery of the method

The analytical recovery was analysed by external standard technique. To mobile phase /drug-free chicken plasma, known concentrations of CTC were added to yield the concentrations of 0.1, 1 and 10.0 $\mu g.ml^{-1}$. Liquid–liquid extraction was used for extraction. The standards were subjected to liquid-liquid extraction. Plasma and mobile phase standards were analyzed as described above. Recovery for plasma standards was calculated as a ratio of the peak area obtained for plasma-based standards and those for mobile phase-based standards. For each concentration, three determinants were made. The percent recovery was calculated according to the regression formula,

$$\textbf{\% recovery} = \frac{n\sum XY - (\sum X)(\sum Y)}{n\sum X2 - (\sum X)2}$$

Where, X= Concentration of drug spiked

Y= Concentration found by assay method (recovery × concentration spiked)

n = number of observations

Precision of the assay method

The precision of the analytical method was determined by evaluating intra-day and inter-day variation. Three different concentrations of the standards were assayed as described above on the same day at different times (intra-day) or on different dates (inter-day). The precision of the method was expressed as coefficients of variation.

RESULTS AND DISCUSSION

A sensitive assay method for the analysis of drugs in plasma and tissues is highly essential for pharmacokinetic studies and residue monitoring. There are several methods for analysis and each has its own merit and demerits. Many authors have reported different methods for the analysis of chlortetracycline including fluorometry assay (Feldman *et al*., 1957), bioluminescent biosensor assay (Virolainen *et al.,* 2008),

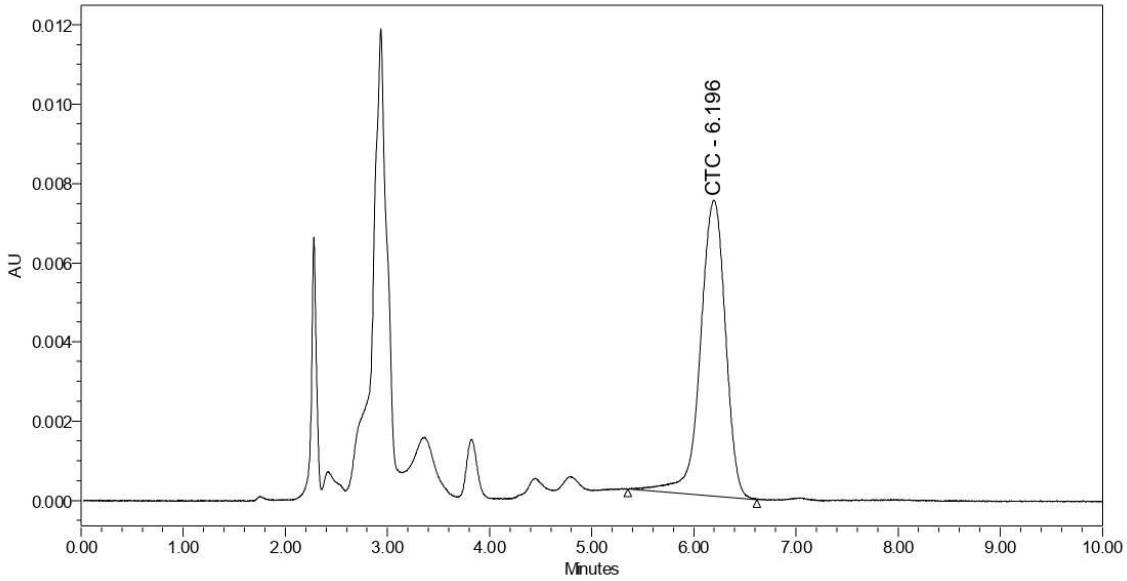

Fig. 1: Chromatogram of CTC in chicken plasma

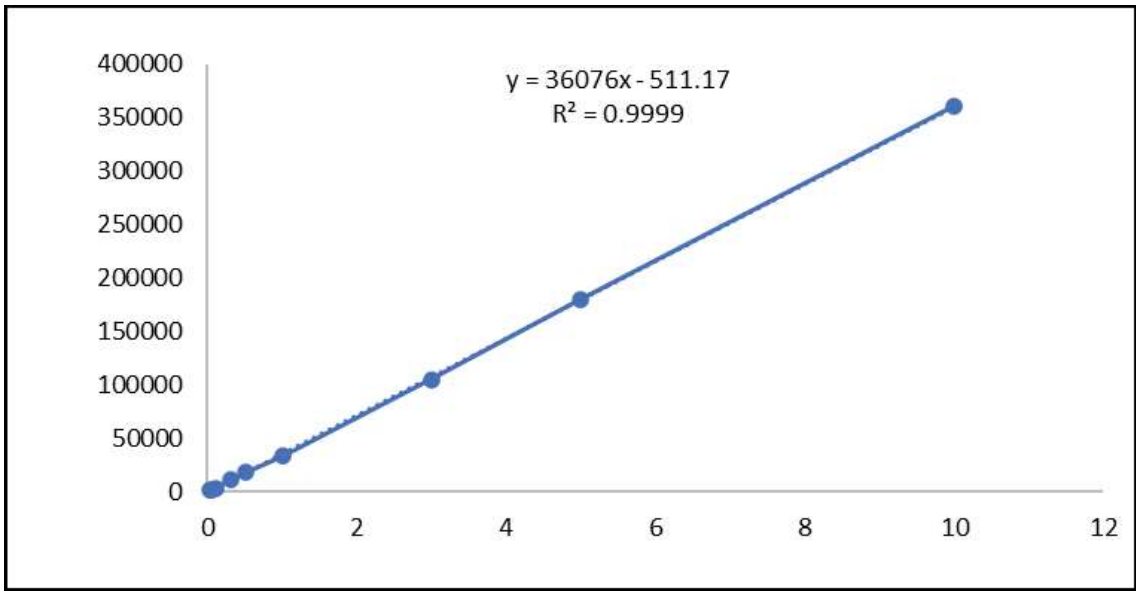

Fig. 2: Standard curve of CTC in mobile phase

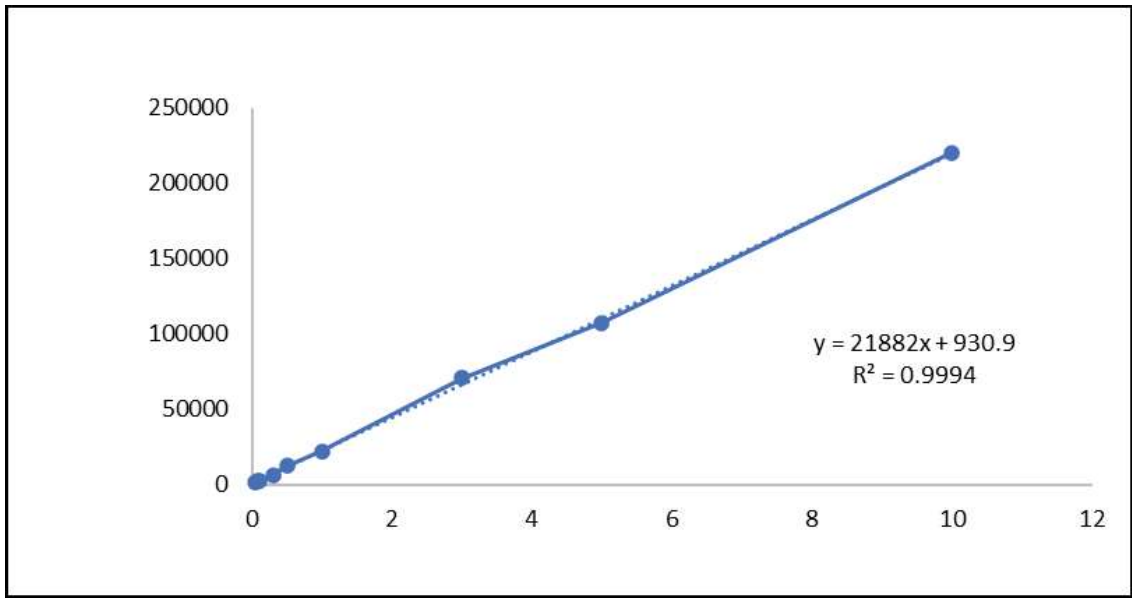

Fig. 3: Standard curve of CTC in chicken plasma

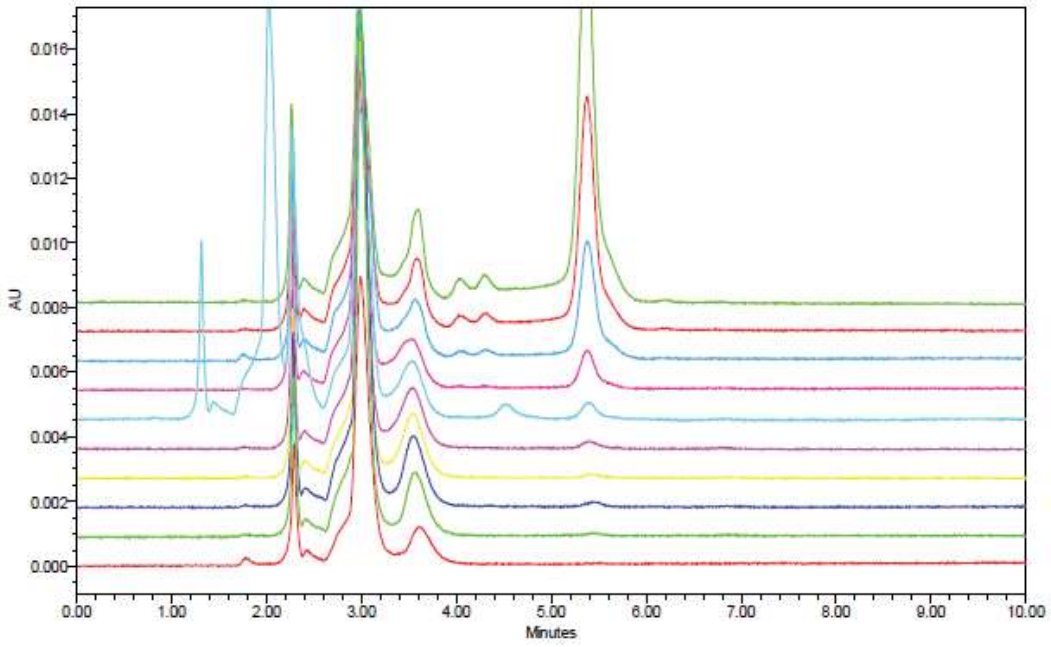

Fig. 4: Overlay of standard curve of chicken plasma –
(Inner box showing increasing peak areas for increasing concentrations of CTC)

Table 1: Intra-day and Inter-day precision of CTC in chicken plasma

Concentration (µg/mL)	Peak area			Mean	S. D	% RSD	Mean (%)
	I	II	III				
Intra-day precision							
0.1	1396	1365	1234	1331.667	85.99031	6.457	3.78
1	9933	10172	10658	10254.33	369.446	3.602	
10	121643	120682	123758	122027.7	1573.665	1.289	
Inter-day precision							
0.1	1149	1109	1259	1172.333	77.67453	6.6256	3.65
1	12777	13537	13406	13240	406.2844	3.0686	
10	149697	148742	152419	150286	1907.95	1.2695	

Table 2: Recovery of CTC in chicken plasma

Concentration spiked (µg/ml)	Concentration found (range, µg/ml)	Recovery (range)	Mean ± S. E.	% Recovery
0.1	0.114 – 0.121	1.139 - 1.209	1.183 ± 0.022	110
1	1.093 – 1.173	1.093- 1.173	1.128 ± 0.023	
10	9.700 –12.192	0.97 -1.219	1.110 ± 0.73	

microbiological assay (Kunin and Finland, 1958; Dornbush and Abbey, 1972 and Stanley *et al*., 1970), high pressure/performance liquid chromatography (Pollet *et al*., 1983 and Reinbold *et al*., 2010).

Even though different analytical methods are available, HPLC has advantages over others due to its high sensitivity, precision, speed, wide range of applications, cost-effectiveness and speed. Microbiological assay techniques cannot precisely determine compounds based on chemical structure and they lack sensitivity and specificity. In this study, a sensitive HPLC method was standardized and validated for the determination of CTC in chicken plasma.

A mixture of aqueous oxalic acid, acetonitrile and methanol was the mobile phase used in this study for CTC assay in chicken plasma samples. This similar combination of mobile phase at a flow rate of 1ml/min with different ratios was reported by Anadon *et al.* (2012).

Various mobile phase combinations were attempted in this study to bring about better elution of the drug. The mobile phase with a mixture of oxalic acid (0.01M),

acetonitrile and methanol at 64:18:18 (Ranwang *et al*., 2010) and 0.2M oxalic acid: acetonitrile: methanol at 3:1:1 v/v/v were attempted and appreciable sensitive specific peaks of CTC was not observed. However, the mobile phase with aqueous oxalic acid (0.03M), acetonitrile and methanol in the ratio of 60:30:10as reported by Shalaby *et al*. (2011) with pH adjusted to 1.84 using 0.1N HCl yielded appreciable sensitive specific peaks of CTC.

In this method, an isocratic elution is used which is better than gradient elution. Isocratic elution is cost-effective and simple for the analyte separation, and there is no need to re-equilibrate with the initial mobile phase between consecutive sample injections (Schellinger and Carr, 2006).

The retention time of CTC was 6.0-6.50 min and the chromatogram of CTC in chicken plasma is shown in Fig. 1. There were no other interfering peaks at the retention time of CTC which shows that this method was highly specific. The shorter retention time of this method is advantageous for analysing a larger number of samples.

The sensitivity of the assay as measured by LOD and LOQ were 0.01 and 0.05 µg/ml, respectively. The reported MIC of CTC against *Mycoplasma sp.* was 0.2 µg/ml (Ricardo *et al*., 2005; Gautier-Bouchardon, 2018). Thus, this method is suitable for assessing the concentration of CTC well below the MIC range.

The linearity of the method was found by plotting the standard calibration curves of spiked chicken plasma samples. The method was linear in the range of 0.05 – 10 $\mu g.ml^{-1}$with a correlation coefficient of 0.998 (Fig. 2, 3 & 4).

The precision of the analytical method was determined at two levels, repeatability (intra-day precision) and intermediate precision (inter-day precision), at three different concentrations (0.01, 0.1 and 10 µg/ml), and the results are given in Table 1. The coefficient of variation was less than 10%, indicating excellent precision of the method. To consider that the analytical method is precise, intra-day and inter-day precision should be < 15% (ICH (Q2) Guidelines, 2005; EMA Guidelines, 2009). The intra-day and inter-day precision of the method was less than 10% and it was well within the limits prescribed by ICH (Q2) guidelines.

The ability of the assay procedure to extract CTC from the plasma matrix is expressed in terms of recovery. The recovery for CTC from plasma was 110% and it was in the acceptable range of 80-120% as per the ICH (Q2) Guidelines, 2005. In the study reported by Ewelina *et al*.(2012), the recovery % of CTC in the medicated stuffs was 93.1%. The variations in extraction techniques could be the reason for variations in recovery.

In conclusion, a simple isocratic HPLC assay method for chlortetracycline estimation in chicken plasma has been suggested with desirable validation parameters. This method can be widely used for PK studies for quantification of CTC in chicken plasma. This can also be attempted for the quantification

of CTC in plasma of other species and also in meat.

ACKNOWLEDGEMENT

The authors would like to thank Tamil Nadu Veterinary and Animal Sciences University, Chennai and the Indian Council of Agricultural Research, New Delhi, for providing the necessary facilities to complete this work.

REFERENCES

Anadon, R., Gamboa, F., Martinez, M.A., Castellano, V., Martinez, M., Ares, I., Ramos, E., H. Suarez, F.H. and Martinez-Larranaga, M.R. (2012). Plasma disposition and tissue depletion of CTC in the food producing animals, chickens for fattening. *Journal of Food Safety,* **18:** 297 – 319.

Dornbush A. and Abbey, A. (1972). Microbiological assay of the tetracyclines. *In Analytical Microbiology*, Academic Press, 365 – 383.

Ewelina P., Ewelina, K. and Krzysztof, K. (2012). Determination of chlortetracycline and Doxycycline in medicated feeding stuffings by liquid chromatography. *Bulletin of the Veterinary Institute in Pulawy,* **56:** 329 - 333.

Feldman D.H., Kelsey H.S. and Cavagnol, J.C. (1957). Fluorometric Determination of Chlortetracycline. *Analytical Chemistry*, **29** (11): 1697- 1700.

Gautier-Bouchardon, A.V. (2018). Antimicrobial resistance in *Mycoplasma* spp., *Microbiology Spectrum,* **6**(4): ARBA030.

ICH Harmonised Tripartite Guideline, (2005). Validation of analytical procedures: text and methodology. International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use.1-13.

Kunin C. M. and Finland, M. (1958). Demethyl chlortetracycline. A new tetracycline antibiotic that yields greater and more sustained antibacterial activity. *New England Journal of Medicine*, **259**(21): 999 - 1005.

Pollet R.A., Glatz, C.E., Dyer, D.C. and Barnes, H.J. (1983). Pharmacokinetics of chlortetracycline potentiation with citric acid in the chicken. *American Journal of Veterinary Research*, **44**(9): 1718 - 1721.

Ran Wang, Ruicheng Wei, Ming Chen and Tian Wang, (2010). A new, simple and rapid HPLC method for determination of chlortetracycline in pig solid manure. *Italian Journal of Animal Science*, **9**: 2.

Reinbold J.B., Coetzee, J.F., Gehring, R., Havel, J.A., Hollis, I.C., Olson, K.C. and Apley, M.D. (2010). Plasma pharmacokinetics of oral chlortetracycline in group fed,

ruminating, Holstein steers in a feedlot setting. *Journal of Veterinary Pharmacology and Therapeutics*, **33**(1): 76 - 83.

Rosenbusch, R.F., Kinyon, J.M., Apley, M., Funk, N.D., Smith, S. and Hoffman, L. J. (2005). *In vitro* antimicrobial inhibition profiles of *Mycoplasma bovis* isolates recovered from various regions of the United States from 2002 to 2003. *Journal of Veterinary Diagnostic Investigation,* **17**: 436 – 441.

Shalaby A.R., Nadia, A., Salama, S.H., Abou-Raya, Wafaa H. Emam and Mehaya, F.M. (2011). Validation of HPLC method for determination of tetracycline residues in chicken meat and liver. *Food Chemistry,* **124**(4): 1660 – 1666.

Schellinger, A.P. and Carr, P.W. (2006). Isocratic and gradient elution chromatography: A comparison in terms of speed, retention reproducibility and quantitation. *Journal of Chromatography A*, **1109**: 253 – 266.

Smith, J. L., Drum, D.J.V., Dai, Y., Kim, J.M., Sanchez, S., Maurer, J.J., Hofacre, C.L. and Lee, M.D. (2007). Impact of antimicrobial usage on antimicrobial resistance in common *Escherichia coli* strains colonizing broiler chickens. *Applied and Environmental Microbiology*, **73**(5): 1404 - 1414.

Stanley E. Katz and Carol A. Fassbender. (1970). Microbiological assay with increased sensitivity for chlortetracycline in eggs. *Journal of Agricultural and Food Chemistry*, **18**(6): 1165 – 1167.

Van Boeckel T. P., Brower, C., Gilbert, M., Grenfell, B.T., Levin, S.A., Robinson, T.P., Teillant, A. and Laxminarayan, R. (2015). Global trends in antimicrobial use in food animals. *Proceeding of National Academy Sciences,* **112**(18): 5649 - 54.

Virolainen E., Pikkemaat, G., Alexander Elferink, J.W. and Karp, T. (2008). Rapid detection of tetracyclines and their 4-epimer derivatives from poultry meat with bioluminescent Biosensor Bacteria. *Journal of Agricultural and Food Chemistry*, **56**(23): 11065 - 11070.