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SPE-HPLC Determination of Doxycycline in Commercial Chicken Meat

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Abstract: Meat producers have fed various veterinary antibiotics to food animals for years in order to prevent bacterial infections and also to promote growth, increase feed efficiency and decrease waste production. However, there are negative effects of these to humans, who consume meat regularly, especially since an inevitable side effect of the overuse of antibiotics is the emergence of drug-resistant bacteria and illnesses. In this study, the presence of doxycycline in raw and boiled commercial chicken meat was determined. Eleven chicken samples were tested: 10 caged and 1 free-range. These were brought from a supermarket along Taft Avenue, Manila. The first batch of chicken meat samples showed distinct antibiotic peaks at around 9 to 10 minutes with LOD and LOQ of 0.15807 ppm and 0.47900 ppm, respectively while the second batch chicken meat samples showed very low peak heights and areas with LOD and LOQ of 0.25215 ppm and 0.76409 ppm, respectively. Seven of 10 raw chicken samples exhibited higher antibiotic concentrations than the regulation standards set by JFCRF and EU. Once the chicken samples were boiled however, the antibiotic concentrations present passed the regulation standards set by the JFCRF and the EU, thus, subjecting raw chicken to boiling decreases its antibiotic residues to acceptable levels set by international standards.

Key Words: *antibiotic resistance; tetracycline; chicken meat; HPLC analysis*

1. INTRODUCTION

Tetracyclines are very important antibacterial compounds commonly used in both medical and veterinary practices. Tetracycline antibiotics are effective against Gram-positive cocci, some Gram-negative cocci, Chlamydia and Mycoplasma. These antibiotics are absorbed well into the body after administration, and are extensively distributed to the lungs, liver and kidney. Therefore, antibiotics are used to prevent and treat diseases of food-producing animals.

Worldwide, there is a growing concern about the prevalence of antibiotic resistance. It is a widely accepted fact that the increase in the use of antibiotics is the main risk factor for the resistance of pathogenic bacteria. This occurrence has led to the emergence of resistant bacteria and illness (van den Bogaard & Stobberingh, 2000). The poor monitoring and testing on the presence of antibiotic residues in broiler meats and lack of published scientific information on the prevalent chemical residues present in commercial meat in supermarkets, has lead the researchers to study commercial meat from these places. Using High Performance Liquid Chromatography, the determination of the presence

and concentration of antibiotics residues in meat is made possible, and the results may be correlated to known limits for human consumption.

The consumption of small amounts of antibiotics on a daily basis could lead to the accumulation of antibiotics within the body and the emergence of drug-resistant bacteria. The analysis of local chicken samples would allow the consumers and distributors of meat to attain more information and knowledge about the meat that they are consuming and distributing.

Antibiotic resistance is the ability of bacteria to resist the effects of antibiotics. This occurs when bacteria modify in a manner which reduces the effectiveness of the drug. Infections with resistant organisms are difficult and costly to treat. (CDC, 2013)

In livestock, tetracyclines are used as veterinary medicine in treating infections in poultry, cattle, sheep, and swine (Chopra and Roberts, 2001). In cases of large numbers of poultry on commercial farms are needed for treatment, the antibiotics are directly added to the feed or water (Chopra and Roberts, 2001). Additionally, tetracyclines are also used in treating infections in domestic pets (Chopra and Roberts, 2001).

According to the CDC, in order to slow down the emergence and spread of antibiotic resistance, responsible antibiotic stewardship must be practiced. Stopping the use of inappropriate antibiotics in humans and animals would help in slowing down the emergence of drug resistant bacteria.

2. METHODOLOGY

2.1 Materials and Equipment

Doxycycline standards were supplied by a veterinary drug store with 98% purity. Methanol (CH3OH) and acetonitrile (C2H3N) were of HPLC-grade by RCI labscan. Citric acid monohydrate (HOC(COOH)(CH2COOH)2H2O), disodium phosphate (Na2HPO4), oxalic acid (C2 H2O4) and ethylene-diaminetetraacetic acid disodium salt (Na2EDTA) were obtained from Sigma-Aldrich and were of analytical grade. Water used was Wilkins distilled water. Solid phase extraction (SPE) of Agilent bond elute C18 cartridge was used for the cleanup of extracts. The HPLC system of Agilent technologies 1200 series equipped with an auto-sampler set at 15-µL, quaternary pump and a diode array detector was used. A reversed-phase analytical column, Restek pinnacle II C18. (250x4.6mm, 5µm) was used. The oven temperature was set at 25°C and UV wavelength was set to detect at 350nm. A flow rate of 1-mL/min was used. Clay Adam compact II centrifuge (3400 rpm), ultrasonic bath, and blender were used for sample preparation.

2.2 Optimization of Parameters

Parameters used for the HPLC analysis were based from a previous validation study done on HPLC parameters for tetracyclines (Shalaby, Salama, Abou-Raya, Emam, & Mehaya, 2011) and was modified and optimized by changing column from a Nuclosil 100 C18 to a Restek pinnacle II C18 and injection volume from 6µL to 15µL.

2.3 Sample Preparation

Chicken meat samples were cut and weighed at 5.00 ± 0.05g into a 50-mL beaker. Two blank chicken samples were spiked with a combined working tetracycline stock solution 100-µL of a 25ppm. Twenty milliliters McIlvaine Buffer-EDTA solution were added into the beaker. Contents were homogenized in a blender for 30 seconds, then transferred back into the beaker plus washings. Samples were then transferred into labeled 15-mL centrifuge tubes. Each sample only used one centrifuge tube. Capped tubes were then centrifuged for 10 mins at 3200 rpm. The supernatant was poured into a second beaker. The process was repeated until all the contents from the initial homogenization had been centrifuged.

2.4 Solid Phase Extraction (SPE)

Agilent bond elute C18 cartridge was used for the cleanup of extracts. The SPE cartridge was conditioned with 20-mL methanol followed by 20-mL water then the test samples. The eluate was discarded and the flash was cleaned. The set up was prepared again to elute the tetracyclines. 6.0-mL 0.01M methanolic oxalic acid was added in to the cartridge. The eluate was then transferred into a 10-mL volumetric flask and diluted to 10-mL with water.

3. RESULTS AND DISCUSSION

Doxycycline is a member of the tetracycline group of antibiotics, commonly used to treat respiratory and urinary tract infections in poultry. Doxycycline is a bacteriostatic compound which behaves by inhibiting protein biosynthesis after binding to the 30 S ribosomal subparticle in a bacterial cell. Doxycycline is more lipids soluble compared to other tetracyclines, thus, it penetrates into body tissues and fluids in a greater extent after its introduction. The strong lipophilic behavior may result in the long persistence of doxycycline in poultry, which can lead to large concentrations of doxycycline in poultry tissues (Chopra and Roberts, 2001). The improper use and administration of doxycycline in chicken can leave behind large amounts of residues, that can be dangerous to human health and may give rise to the resistance of many microorganisms to Doxycycline.

3.1 Calibration Curve

Doxycycline registered a single strong signal with a retention time of approximately 10 minutes. Standard calibration curve was done for doxycycline using concentrations of 5ppm, 2.5ppm, 1ppm, 0.5ppm, 0.1ppm, 0.05 ppm and 0.01ppm. R value obtained from doxycycline standards is 0.9812, revealing a satisfactory calibration curve for determining the concentrations in unknown samples. A similar calibration curve with identical range of standard concentrations was used in a previous study (Ang, Estaniel and Guidote). Like in the said study, Doxycyline concentrations found in commercial chicken fall within this range. Equation of the line of the standard curve is y = 35.705 x – 8.185. Fig 1 shows the doxycycline calibration curve.

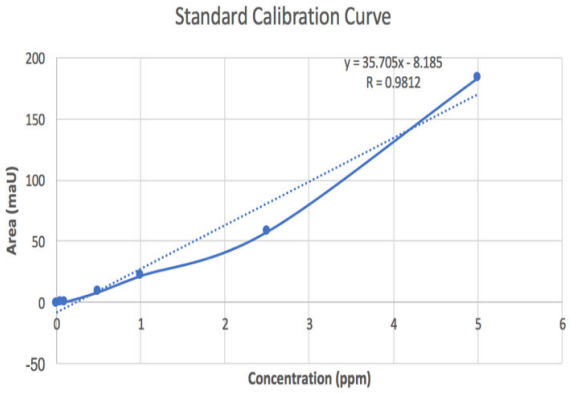

Fig 1. Standard Calibration Curve for Doxycycline

Another standard calibration curve was constructed from the obtained peak areas of the working stock solutions for the second batch of analysis. Since the chromatograms for the second batch of samples showed a distinct difference (decreased) in retention time, peak heights, and peak areas, another calibration curve was constructed in order to account for the changes exhibited by the chromatogram for the second batch of samples analyzed. Concentrations from 5.00 ppm, 2.50 ppm, 1.00 ppm, 0.500 ppm, 0.100 ppm and 0.0500 ppm were considered in constructing the calibration curve. The equation of the line is y = 23.017 x + 6.5329 with a correlation coefficient (R) of 0.9898. The limit of detection (LOD) and limit of quantitation (LOQ) where calculated to be 0.25215 ppm and 0.76409 ppm, respectively. LOD and LOQ values were arrived at using the equations:

$$\text{Detection limit, LOD} = \frac{3s}{m}$$

$$\text{Quantitation limit, LOQ} = \frac{10s}{m}$$

where s is the standard deviation and m is the slope of the calibration curve. LOD reports the minimum detectable concentration of the analyte. On the other hand, LOQ pertains to the smallest amount of the analyte that is measureable with reasonable level of accuracy.

3.2 Analysis of Chicken Meat Samples

A total of 11 chicken samples were analyzed for doxycycline content. Among the 11 samples, one free-range chicken was analyzed as a control and ten were caged chicken. The ten caged chicken samples were then analyzed when these were raw and then boiled. Based on chemical analysis performed, the control (or free-range) chicken exhibited a concentration of 0.09594 ppm. Although free-range or organic chickens are marketed as having no antibiotics directly administered to them, the results of this study suggest otherwise. The results of this study are in line with previous findings done by Ang, Estaniel and Guidote (2016), wherein the free-range chicken analyzed in their study, displayed a concentration of 0.5566 ppm. Therefore, the free-range chicken analyzed in this present study, contains much less Doxycycline residues compared to the previous study conducted. There are several possible ways for which the detected antibiotic residues would have indirectly reached the free-range chicken. First, it is possible that the environment (soil, air, waterways, etc.) wherein the free-range chicken was raised in may have been contaminated with antibiotics, especially if chicken raised in conventional manner are also raised within the same farm. Second, the source of water through which the free-range chicken may have drunk from may have already contained antibiotics. Third, it is also plausible that the food and vitamins being administered to the free-range chicken may have also contained antibiotic residues, thus, allowing the free-range chicken to be exposed to the drug. Fourth, there is the possibility of contamination due to human errors or farm workers who are not too careful in caring for their stock.

Table 1 summarizes all the important doxycycline results from the HPLC chromatogram for the raw chicken samples with a control assumed positive for doxycycline. The chicken samples with concentrations labeled as N/A means that their concentrations are less than 0.05ppm, the lowest concentration of the standard used to construct the calibration curve.

Table 1. Analysis of Doxycycline via HPLC

RAW CHICKEN SAMPLE	BATCH	PEAK AREA (mAU)	CONCENTRATION (ppm)
BF1	1	2.10076	0.2915
BF2	1	0.063900	0.2355
BF3	1	0.815520	0.2551
BF4	1	0.0312100	0.2327
BF5	2	8.06533	0.06658
MG1	1	4.60418	0.3614
MG2	1	3.64291	0.3334
MG3	2	1.77597	N/A
MG4	2	5.25357	N/A
MG5	2	1.76479	N/A
Control (Free-Range)	1	-4.75937	0.09846
Blank	1	0.00000	0.000

Figure 2 shows that comparing the results of this study with the Maximum Residue Limit (MRL) of 0.1 ppm as set by the EU International Regulation Standard and 0.05 ppm for the Japan Food Chemical Research Foundation, the present study suggests that the samples used herein contain doxycycline in concentrations well beyond the acceptable residue limit for doxycycline in chicken muscle.

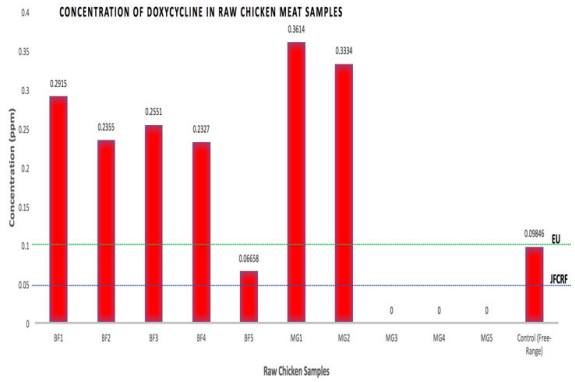

Fig 2. Concentration of Doxycycline in Raw Chicken compared to MRLs set by international standards

Table 2 summarizes all the important doxycycline results from the HPLC chromatogram for the boiled chicken samples. The chicken samples with concentrations labeled as N/A means that their concentrations are less than 0.05ppm, the lowest concentration of the standard used to construct the calibration curve.

Table 2. Analysis of Boiled Chicken Samples

BOILED CHICKEN SAMPLE	BATCH	PEAK AREA (mAU)	CONCENTRATION (ppm)
BF1	2	2.1451	N/A
BF2	2	1.8033	N/A
BF3	2	N/A	N/A
BF4	2	2.38352	N/A
BF5	2	5.68412	N/A
MG1	2	N/A	N/A
MG2	2	N/A	N/A
MG3	2	N/A	N/A
MG4	2	N/A	N/A
MG5	2	N/A	N/A

Although no concentrations were calculated from the boiled chicken samples due to the very low peak heights and peak areas the chromatograms of these boiled chicken samples exhibit, it can be said that since their peak areas are smaller than that of the peak area of the lowest concentration of the Doxycycline standard, 0.0500 ppm, used to construct the calibration curve, then their concentrations must be lower than 0.0500 ppm. These results are in agreement with a previous study conducted by Abou-Raya, et al. (2013), wherein the concentrations of Doxycycline in raw chicken samples decreased by 27.7% when cooked by boiling for 30 minutes. Thus, subjecting raw chicken meat to boiling would lead to a considerable decrease in Doxycycline antibiotic concentration, rendering the boiled chicken meat samples safe for human consumption based on the permissible limits set by the EU and JFCRF.

4. CONCLUSIONS

This study shows that Doxycycline residues in chicken is present in substantial amounts. The present analysis showed that Doxycycline concentrations ranging from 0.06658 ppm to 0.3614 ppm is present in raw chicken. These Doxycycline concentrations are generally beyond the Maximum Residue Limits set by EU and JFCRF. This study also showed that boiling of meat removes a considerable amount of the Doxycycline residue. Although the fate of the antibiotic after boiling is unclear, for it could have been degraded, extracted into the chicken stock, this study showed that the Doxycycline antibiotic was no longer present in the meat or is present at a very low concentration. Although the calculated concentrations of Doxycycline in this study are not as large compared to the previous study conducted by Ang, Estaniel and Guidote (2016), the trend remains consistent that the Doxycycline residues in local poultry are way beyond the accepted maximum residue limits set by international standards. Since chicken is a common food consumed by Filipinos, there is a real threat of the accumulation of Doxycycline in the human body, which can ultimately lead to antibiotic resistance.

5. ACKNOWLEDGMENTS

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6. REFERENCES (use APA style for citations)

Abou-Raya, S. H., Shelby, A. R., Salama, N. A., Emam, W. H., & Mehaya, F. M. (2013). Effect of Ordinary Cooking Procedures on Tetracycline Residues in Chicken Meat. Effect of Ordinary Cooking Procedures on Tetracycline Residues in Chicken Meat, 21(1), 80-86. doi:10.6227/jfda.2013210110

Ang, J. D., Estaniel, S. S., & Guidote, L. P. (n.d.). Extraction and Determination of Tetracyclines in Chicken Meat by Solid Phase Extraction and High Performance Liquid Chromatography. Retrieved December 27, 2017.

Connell, S. R., Tracz, D. M., Nierhaus, K. H., & Taylor, D. E. (2003, December). Ribosomal Protection Proteins and Their Mechanism of Tetracycline Resistance. ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, 47, 3675-3681.

Chopra, I., & Roberts, M. (2001, June). American Society of Microbiology. Tetracycline Antibiotics: Mode of Action, Applications, Molecular Biology, and Epidemiology of Bacterial Resistance, 65, 2nd ser., 232-260. Retrieved October 20, 2016, from Antimicrobial Research center and Division of microbiology.

Shalaby, A., Salama, N. A., Abou-Raya, S., Emam, W. H., & Mehaya, F. (2011). Validation of HPLC method for determination of tetracycline

residues in chicken meat and liver. *Food Chemistry*, *124*(4), 1660-1666. doi:10.1016/j.foodchem.2010.07.048

Van den Bogaard, A.E. and Stobberingh, E.E. (2000) Epidemiology of Resistance to Antibiotics. Links between Animals and Humans. International Journal of Antimicrobial Agents, 14, 327-335. http://dx.doi.org/10.1016/S0924-8579(00)00145-X

Webber, M., & Piddock, L. (2003). The importance of efflux pumps in bacterial antibiotic resistance. Journal of Antimicrobial Chemotherapy, 9-11.