Screening of Some Antibiotics and Anabolic Steroids Residues in Broiler Fillet Marketed in El-Sharkia Governorate

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Abstract: Antibiotics and anabolic steroid growth promoters usage have 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. Therefore, this study was carried out on one hundred randomly collected fresh and frozen broiler fillet samples (50 of each) to evaluate the antibiotic residues level qualitatively by microbiological inhibition assay followed by quantitative detection for oxytetracycline and enrofloxacin by high performance liquid chromatography (HPLC). In addition to monitoring of anabolic steroids (Testosterone, Progesterone and Zeranol) quantitively by Enzyme- linked immunosorbent assay (ELISA). The obtained results revealed a detectable level of oxytetracycline residues which confirm widespread misuses of antibiotic especially oxytetracycline in farms and lack of application of recommended withdrawal times. The anabolic steroids residues level including testosterone and progesterone were within the permissible limit which refers to no illegal use of hormones as growth promoting agents in broiler production. None of the samples displayed the presence of zeranol residue as the level of it was below the detection limit of the used kits (<100 ng/kg). For monitoring the effect of cooking process on antibiotic residue levels in broiler fillet, ten broilers (40 days old) were classified into two groups, each group was dosed over 5 consecutive days with 15 mg/ kg day of oxytetracycline or enrofloxacin. Five slices of broiler fillet from each group subjected to cooking either via frying or grilling.The results showed that cooking had an effect in reducing the concentration of antibiotic residues as there are a signficant reduction percentages of oxytetracycline while enrofloxacin residues showed low reduction percentages. These findings recommended that restricted measures and harder regulations must be applied to prevent the misuse of drugs in poultry industry, application of withdrawal time as well as the inspection of chickens for drug residues prior to marketing.

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1. Introduction

Poultry products are important protein sources. For this purpose, birds are reared as broilers for meat and layers for eggs, under intensive or free range management. In Egypt, broiler meat production was 559,000 tones, representing 84% of total poultry meat production **(Maged and Hamdey 2006)**. Demands for high quality parts and further processed convenience foods have driven the poultry industry to change its marketing practices. Primarily poultry was sold in supermarkets as ready-to-cook whole carcasses. Today, boneless fillet have become critical to processors, yield of fillet (Boneless, skinless pectoralis major) ranged from 14.9 to 15.1% **(Young et al., 2001)**.

The administration of health-risk related substances such as growth promoting agents and veterinary drugs (antibiotics) is a recurring problem in animal production where these compounds are often used to increase the productivity and to reduce breeding costs **(Toffolatti et al., 2006)**. Uses of them in broiler chicken farms for therapeutic and performance-enhancing purposes may lead to deposit of residuals in their carcasses, particularly when the birds are slaughtered without the observance of withdrawal period of the drug **(Donoghue & Hairston, 2000 and Kan & Petz, 2000)**. Ignorance of observation of withdrawal period leads to a serious threat to human health upon exposure to these residues. Therefore, residues monitoring are required in detecting anabolic and veterinary drugs treatments for the safety of consumers.

Antibiotics are used extensively in poultry industry for the treatment and prevention of several diseases, as well as to improve feed efficiency and promote growth **(McEvoy 2002 and Di Corcia & Nazzari, 2002)**. In addition assist in converting stress due to environmental changes, vaccination, debeaking and other management practices. This wide spread use of antibiotic may cause residuals in foodstuffs, as well as the induction of allergic reactions in humans. In addition, resistance to pathogenic bacteria has been

constantly weakened as a result of antibiotic usage **(Schenck & Callery, 1998)**. Oxytetracycline is a natural tetracycline compound that is derived from the fungus, *Streptomyces rimosus*. 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 **(Slana & Dolenc, 2013)**. 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 **(Unisol, 2010)**. Many endogenous steroids, including their semi-synthetic and synthetic analogues, have been produced and administered to animals to improve growth of animals for food production, as well as to regulate and enhance fertility **(Lone, 1997)**. Some scientific reports stated their possible carcinogenicity, genotoxicity and interfere with human and animal natural physiological function. Also, it has been found that the highest rates of hormone-related cancer, including cancer of breast, ovary, prostate, testes and colon were found where hormone-treated meat is consumed **(Andersson & Skakkebaek, 1999 and Sibbald, 1999)**. In light of the carcinogenic potential of their residues and obvious human health risks, the European Community forbade the use of steroids as growth-promoting agents in livestock breeding **(EC, 1996&2010)**. In some countries, a related synthetic estrogenic compound zeranol are officially registered for use as hormonal growth promoting compounds owing to their anabolic and/or partitioning effect because of the potential for toxicity at a very low level, the use of zeranol has been completely forbidden in the European Union. Hence the maximum residue limits (MRLs) of this compound in animal tissues is undetermined **(Fang et al., 2002)**.

The objective of this study is to through the light on safety of the broiler fillet which used in large quantities in homemade or in ready to eat food through residues monitoring of antibiotics (Oxytetracycline & Enrofloxacin) and anabolic steroid growth promoters either natural (Testosterone & Progesterone) or synthetic (the estrogen compound Zeranol), with special refrences to the effect of the most common cooking procedures of it (frying and grilling) on the antibiotic residues level.

2. Materials and methods

One hundred random broiler fillet samples (fresh from small scale production poultry processing shops and frozen from large scale production from the super market, 50 of each) were collected, wrapped in polyethylene bags and put in cool boxes with dry ice or freezer packs. The samples were subsequently rapidly transported under a complete aseptic condition to the laboratory of Food Control Department. Faculty of Veterinary Medicine, Zagazig University, then prepared for antibiotics and anabolic steroid growth promoters residues monitoring.

A. Evaluation of antibiotic residues

Two methods were used simultaneously for the determination of antibiotics residues in broiler fillet.

1. Qualitative evaluation: Microbiological inhibition assay using *Bacillus subtilis* (ATCC-6633) as indicator organism. The level of antibiotics can be evaluated by measuring the diameter of inhibition zone observed on an agar layer seeded with a test organism (with a caliper) according to **Levetzow and Wiese (1979)**. The indicator organism was obtained from Department of Bacteriology, Animal Health Research Institute in Doki, Giza.

2. Quantitative evaluation: HPLC analysis was used for determination of oxytetracycline and enrofloxacin residues level in positive samples resulted from the microbiological inhibition assay.

HPLC analytical procedures

Sample extraction

Fresh and thawed broiler fillet samples were finely sliced after trimming off external fat and fascia.

Oxytetracycline: Two grams of broiler fillet were cutted into very small pieces and subsequently ground into fine particles using Sartorius mincer, then homogenized for 2 min and then 0.1 g citric acid was added. To this mixture, 1 ml nitric acid (30%), 4 ml methanol (HPLC grade) and 1 ml deionized water were added, respectively. The suspension with solid particles was vortexed, kept in an ultrasonic bath for 15 min and centrifuged for 10 min at 4000 rpm. After filtering through a 0.45 µm nylon filter, 20 µl of solution was injected into HPLC for analysis according to **Senyuva et al. (2000)**.

Enrofloxacin: Five grams of broiler fillet were transferred to a 30-ml centrifuge tube, 20 ml of 1 M HCl was added and the mixture sonicated for 5 min. The tube was subsequently centrifuged for 5 min at 4000 rpm. The supernatant was taken after centrifugation and Sep-Pak C_{18} cartridges, previously conditioned, are then used for purification. The cartridges were washed with 10 ml of water and the elution of enerofloxacin was performed with 4 ml of mono potassium phosphate (1 mM), pH 2.5–methanol (1:1) mixture. The purification residues were evaporated in a nitrogen stream at 35º C, 20 µl of solution was injected into HPLC for analysis according to **Gigosos et al. (2000)**.

Chromatographic conditions

Oxytetracycline: The mobile phase consisted of methanol (HPLC grade) and formic acid 0.1% using a gradient method with a flow rate of 1.5 ml/min at 25^{o}C. The separation was done on hypersil gold C_{18} (10 µm, 100x4.6 mm) column with mobile phase. Detection was performed with photodiode array detector (PAD) set at 350 nm wave length. Quantification of residues in samples was obtained and calculated from areas under

curves extrapolated automatically by the software (Chromo Quest 5).

Enrofloxacin: The mobile phase consisted of a mixture of 0.1 M orthophosphoric acid, pH 3.5–acetonitrile (85:15, v/v). The eluent was filtered prior to use. Detection was performed with photodiode array detector (PAD) set at 280 nm wave length at a flow rate of 1 ml/min. Chromatographic separation was achieved on a C_{18} hypersil BDS (5 µm, 250×4.6 mm) column.

The concentration of antibiotics residue in the samples were calculated with reference to a calibration curves obtained from work solutions of oxytetracycline and enrofloxacin ranged from 0.01 to 50 µg/ ml and 0.5 to 10 µg/ ml respectively. For the preparation of the work solutions, Oxytetracycline hydrochloride (Sigma Aldrich, Inc., St. Louis, USA) and Enrofloxacin (Bayer Pharmaceuticals, West Haven, CT, USA) stock solutions (1 mg/ml in methanol) of the antibiotics were diluted to concentrations previously mentioned by using methanol as diluents.

B. Evaluation of anabolic steroid growth promoters

Anabolic steroids residue screening were carried out by using commercial ELISA kits (Art. No. DRG1561, DRG1559 and R3301) specific to progesterone, testesterone and zeranol respectively, obtained from r- Biopharm AG, Germany and stored at 4ºC. Kits were supplied with reagents for the enzyme immunoassay including standards and specific coated micro- titer plates. The sample extraction and estimation were performed based on the manufacturer procedure described by the ELISA kits.The senstivity range of progesterone and testesterone assays were 0.03-0.07 ng/ml, 0.05-0.09 ng/ml respectively and detection limit of the used zeranol kits was (100 ng/kg).

C. Effect of cooking procedures on oxytetracycline and enrofloxacin residues level

Animal treatment

Ten broiler chickens (40 days age with average weight 1.750- 2 kg), classified into two groups. Every 5 broiler housed in identified cage. Each group was dosed over 5 consecutive days with 15 mg/ kg day of oxytetracycline and enrofloxacin. through the intramuscular route. The pharmacological speciality Oxytetracycline 5% and Enroflox (Enrofloxacin 10 %) were obtained from El- Nasr Company for Pharmaceutical Industry.

The chickens were slaughtered after 24 hours from the last dose and dressed to obtain the fillet. Five representative slices of broiler fillet from each group weighting 50 ± 4 g subjected to cooking either via frying (fried for 10 min with 400 ml sunflower oil in a pan, turning occasionally. The cooked fillet had a "well done" appearance on the outside) or grilling (grilled for 10 min). The microbiological inhibition assay method was applied on cooked broiler fillet for monitoring the effect of cooking in antibiotics residues level.

Statistical analysis

Data of the current study was statistically analyzed using the computer program **SPSS/PC (2001)**. The statistical method was one way ANOVA test.

3. Results and Discussion

Antibiotic residues in fresh and frozen broiler fillet

Our results revealed that the 34% from small scale fresh broiler fillet samples were positive for antibiotic residues, with an inhibition zone of 1.82 ± 0.371 mm. While only 8 % from the samples of large scale frozen broiler fillet were positive for antibiotic residues, with an inhibition zone of 0.62 ± 0.24 mm. There are significant difference between the small scale and large scale at ($p<0.01$). The difference may be attributed to the selection of broiler flock after elapsing of the withdrawal time of antibiotic treatment in large scale production, while this selection not occur in small scale production. Not only selection process but also, freezing may be act as a factor in reduction of antibiotic residues in examined frozen samples as previously mentioned by many authors **Mansour (2000), Okerman et al. (2007) and Mahmoud and Mohsen (2008)**.

From the obtained data in Table (1): It was found that the sum of positive samples for antibiotic residues in both fresh and frozen fillet representing 21% of total examined samples. These results were nearly in accordance with the results obtained by **Shahid et al. (2007)** which represent 20.4% in examined muscle samples. While higher percentages were detected by **Mahmoud and Mohsen (2008)** and **Shareef et al. (2009)** as they recorded the antibiotic residues in 50 and 56% respectively from the analyzed breast muscles.

The positive samples resulted from the microbiological inhibition assay were analyzed by HPLC for quantification of oxytetracycline and enrofloxacin residues. six samples from the 21 analyzed samples (31.5%) revealed a detectable level for oxytetracycline residues in fresh samples. The residual level ranged from 0.156 µg/g to 0.900 µg/g as shown in Figures (1 and 2) with a mean value of 0.394 ± 0.111 µg/g. The results reported in this study were consistent with these previously reported by **Abd El. Monem et al. (2002)** and **Gad (2012)**. Our results were higher than that obtained by **Salehzadeh et al. (2006) and Shahid et al. (2007)**, who recorded a residue level ranged from 0.0066 to 0.2553 and from 0.030 to 0.085 µg/g respectively.

The high residual level of oxytetracycline may attribute to the production of oxytetracycline in different trade names and forms in many companies in Egypt. So the broiler stock holders use it as cheap effective antibiotic for control of infections and as feed additives at sub-therapeutic levels as a growth

promoting agent. Oxytetracycline residues detected in fresh broiler fillet in this study may be due to the destructive effect of freezing on oxytetracycline **(Okerman et al., 2007)**.

Regarding to enrofloxacin residues, there were two samples in the fresh fillet at a level of 0.04 µg/g (Figure 3) and 0.757 µg/g (Figure 4) and one sample in frozen fillet at a level of 0.218 µg/g (Figure 5). The obtained results were higher than those recorded by **Salehzadeh et al. (2007)** as they detect 0.018 µg/g in examined chicken muscle.

The MRLs permitted by the European agency for the evaluation of medicinal products, Committee for veterinary medicinal products, for enrofloxacin and its metabolite ciprofloxacine are 100- 300 µg/kg in muscle, liver and kidney of bovine, porcine, rabbit, ovine and poultry species **(EMEA, 1998)**. European Union commission recommended the MRLs of oxytetracycline and enrofloxacin in the edible tissues of food producing animals were 0.1µg/g **(EC, 2010)**. The results of this study showed indications of violation of these recommendations.

From the results achieved in Table (2) all oxytetracycline positive samples found to be higher than the MRLs, while only 66.7 % of enrofloxacin positive samples were exceed MRLs, this may attributed to the unpaid attention to the withdrawal period of the antibiotic and extra label use. The microbial resistance to antibiotics may arise as result of animal exposure to these agents and this resistance may be transferred to human pathogens **(Yorke and Froc, 2000)**. In addition human exposure to animal products containing significant level of antibiotic residues may prove immunological response in susceptible individuals and cause disorder of intestinal flora **(Zaki et al., 2000)**.

Anabolic steroids residues in fresh and frozen broiler fillet

The results in Figure (6) revealed that the testosterone level in fresh fillet ranged from 0.10 to 0.70 µg /kg with a mean value of 0.33 ± 0.026 µg /kg, while the level in frozen fillet ranged from 0.10 to 0.60 µg /kg with a mean value of 0.28 ± 0.017 µg /kg. Results obtained by **Kadimi et al. (2010)** and **Zeitoun and Ahmed (2011)** revealed higher detectable levels (25.531 µg /kg and 2.008 µg /kg respectively) in chicken meat in Sultanate of Oman and Saudi Arabia. Testosterone levels were not statistically different ($p>0.05$) in fresh than in frozen fillet.

In the present study, the mean progesterone level in fresh fillet were 0.414 ± 0.039 µg /kg, while in frozen fillet were 0.364 ± 0.026 µg /kg. Higher results were obtained by **Zeitoun and Ahmed (2011)**, they detect 4.065 µg /kg in chicken meat at Saudi Arabia. Progesterone levels were not statistically different ($p>0.05$) in fresh than in frozen fillet. The obtained results clear that the detectable testosterone and progesterone level were attributed to endogenous hormones and confirmed that the broiler stock holders in Egypt nowadays don't use hormones as growth promoters.

According to the US-FDA guideline, they allowed incremental increases above the normal levels of progesterone and testosterone in muscle up to 3 and 0.640 µg /kg **(Center for Veterinary Medicine, Food and Drug Administration, CVM-FDA, 1994 a&b)**. Moreover, acceptable daily intake (ADI) of progesterone, and testosterone were established by the Joint Expert Committee on Food Additives (JECFA) at 30 and 2 µg /kg body weight **(Joint FAO /WHO, 1999)**. This mean that human weighting 70 kg can tolerate about 2100 and 44.8 µg of progesterone and testosterone daily, so he can eat fillet in any quantity without complaining of steroid residues, but some *in vivo* studies indicated that even small differences in hormone levels and very low doses of steroid hormones may have significant adverse biological effects **(Caruso-Nicoletti et al., 1985 and Masamura et al., 1997).**

Effect of cooking procedures on antibiotic residues in broiler fillet

The most common methods in cooking of fillet were frying and grilling, so this study evaluate the effect of these methods on the concentration of antibiotic residues by microbiological inhibition method as shown in Figure (7). Frying significantly diminish the percentage of residues of oxytetracycline (95.7 %) more than grilling (91.4 %) while enrofloxacin residues reduction percentages were 25.6 % and 33.3 % for frying and grilling respectively. The effect of cooking method on oxytetracycline residues coincide with **Marouf and Bazalou (2005)**, who studied the effect of frying process and the reported reduction percentage was 85.71%. Also **Rose et al. (1996)** discussed the heat stability of oxytetracycline and found that the drug was unstable. The result of frying and grilling on enrofloxacin residues was nearly similar to that obtained by **Lolo et al. (2006)**. On contrary **Javadi et al. (2011)**, who revealed a significant decrease of enrofloxacin residues. What ever the reduction percentages of the antibiotic residues, not render the fillet safe for human as antibiotic may destructed to harmful metabolites. Only applications of strict measure for maintaining the flocks in the farm till elapsing of the withdrawal period could solve the problem of human exposure to antibiotic residues.

In Conclusion: The widespread misuse of antibiotics especially oxytetracycline in farm and lack of implementation of recommended withdrawal time was ensured. Also, 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.

Table (1): Antibiotic Residues Level in Fresh and Frozen Broiler Fillet by Microbiological Inhibition Assay.

No of samples	No of positive samples	percentages of positive samples	Inhibition zone in mm		
			Minimum	Maximum	Mean ± SE
50 fresh broiler fillet	17	34%	zero mm	11 mm	1.82 ± 0.371
50 frozen broiler fillet	4	8 %	zero mm	5 mm	0.62 ± 0.24

Table (2): Residues Level of Oxytetracycline and Enrofloxacin in Positive Samples in Comparison with Maximum Residue Limits (MRLs) in µg/ g: according to EC (2010).

Antibiotic	Sample No	Residues level (µg/g)	MRLs (µg/g)	Judgment
Oxytetracycline	**1**	0.339 µg/ g	0.1µg/g	**Rejected**
	2	0.439µg/ g		**Rejected**
	3	0.350 µg/ g		**Rejected**
	4	0.156 µg/ g		**Rejected**
	5	0.900 µg/ g		**Rejected**
	6	0.178µg/ g		**Rejected**
Enrofloxacin	**1**	0.04 µg/ g	0.1µg/g	**Pass**
	2	0.757 µg/ g		**Rejected**
	3	0.218 µg/ g		**Rejected**

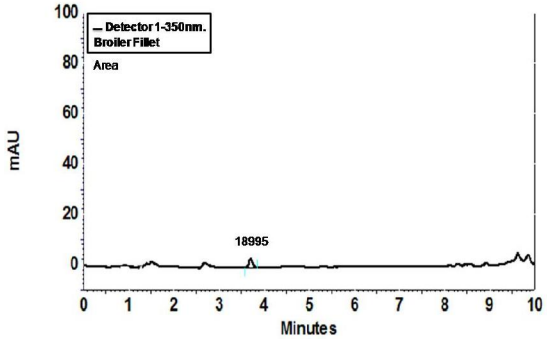

Figure (1): HPLC Chromatogram of Oxytetracycline Residue in Fresh Broiler Fillet.

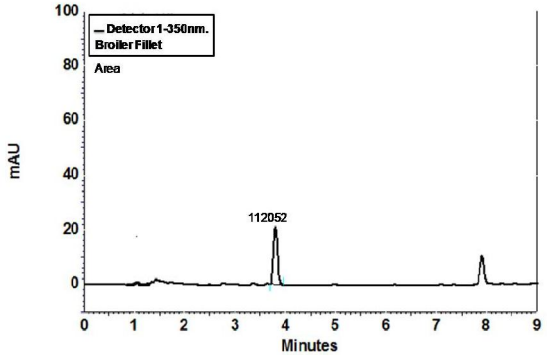

Figure (2): HPLC Chromatogram of Oxytetracycline Residue in Fresh Broiler Fillet.

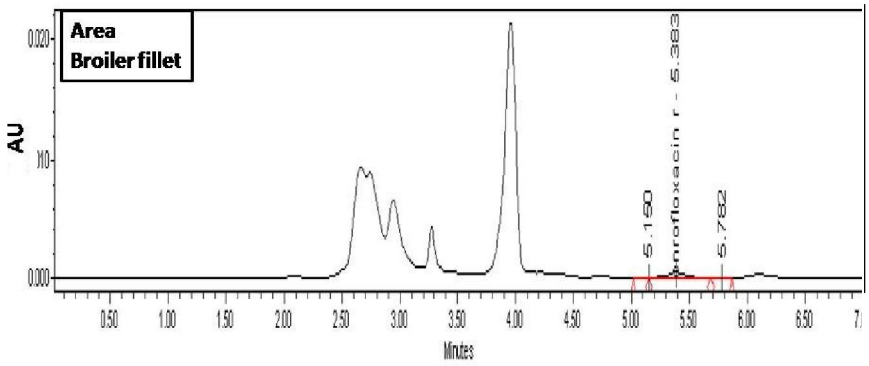

Figure (3): HPLC Chromatogram of Enrofloxacin Residue in Fresh Broiler Fillet.

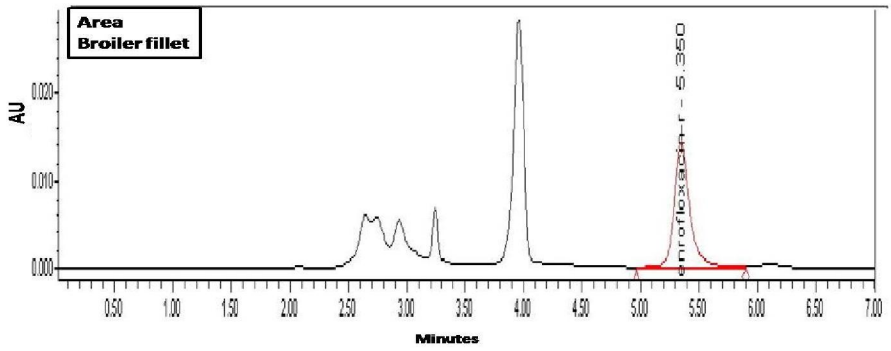

Figure (4): HPLC Chromatogram of Enrofloxacin Residue in Fresh Broiler Fillet.

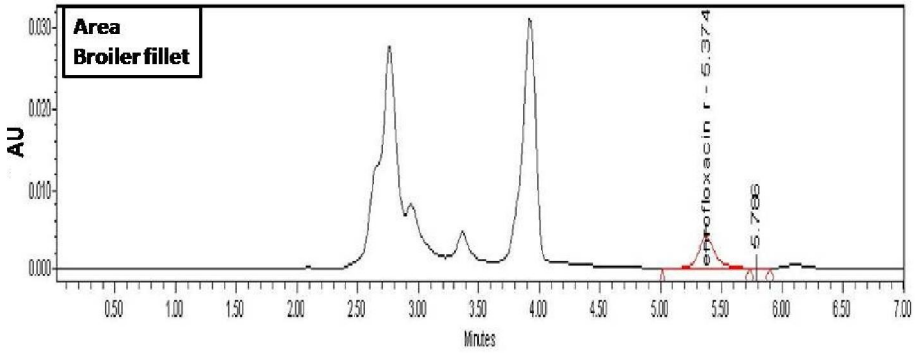

Figure (5): HPLC Chromatogram of Enrofloxacin Residue in Frozen Broiler Fillet.

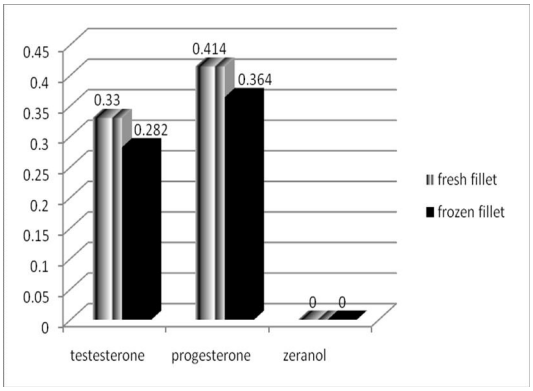

Figure (6): The Mean Detectable Residues Level of Testosterone, Progesterone and Zeranol in Fresh and Frozen Broiler Fillet.

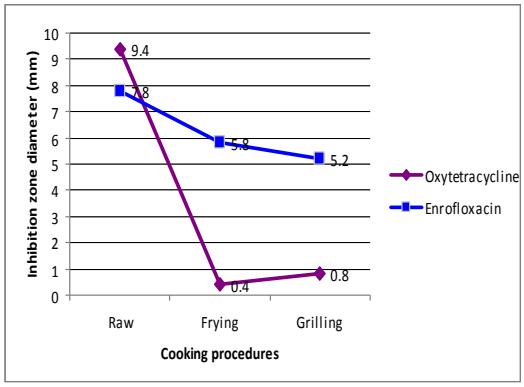

Figure (7): Effect of Different Cooking Procedures on the residual Level of Antibiotic Represented by Inhibition Zone Diameter (mm).

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