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RESIDUES OF SOME FLUOROQUINOLONES IN BREAST, LIVER AND KIDNEYS OF FRESH BROILER CHICKEN CARCASSES

By

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ABSTRACT

The present study was carried out to investigate the presence of the residues of the two most commonly used fluoroquinolones, enrofloxacin and ciprofloxacin in breast muscles, liver and kidney samples of fresh broiler chicken carcasses by using an ultra-performance liquid chromatography coupled to mass spectrometry (UPLC-MS/MS) techniques. The obtained results revealed that two samples were positive for enrofloxacin residues analysis. The first positive sample was for breast muscles and recorded 8.11 µg/kg and it was within the recommended permissible limit of muscles (100 µg/kg) **commission Regulation EU/No.37 (2010)**. Meanwhile, the second positive sample was a kidney sample, which recorded a concentration of 579.6 µg/kg that is higher than the permissible limit for kidney (300 µg/kg) (**Commission Regulation EU/No. 37, 2010)**. On the other hand, results showed that none of the examined samples has residues of ciprofloxacin. The possible public health risks of detected antibiotic residues were discussed.

Keywords:

Fresh broiler chicken, edible offal - antibiotic residues fluoroquinolones, enrofloxacin and ciprofloxacin - UPLC-MS/MS techniques.

INTRODUCTION

Antibiotics are natural, semi-synthetic and synthetic compounds with antibacterial activity that destroy or inhibit the growth of bacteria. In poultry industry, veterinarians to treat bacterial infections, as prophylaxis to reduce the incidence of bacterial diseases, use antibiotics. Increased density of broiler chicken in intensive rearing operations requires a right approach to diseases control, which can lead to heavy prophylactic and proper therapeutic antibacterial use. Antibiotic usage has facilitated the efficient commercial production of poultry as indicated by decreasing mortality, morbidity rates, allowing the consumer to

purchase high quality meat at a reasonable cost **(Tollefson and Miller, 2000; Donoghue, 2003 and Vishnuraj *et al*., 2016)**.

Despite the benefits provided by Antibiotics, overuse and misuse of antibiotics in poultry industry has the potential to produce harmful antibiotic residues in edible poultry tissues (meat and offal) which can lead to adverse health effects on consumers **(Gouvêa *et al*., 2015 and Mund *et al*., 2017)**. Antibiotic residues in foods of animal origin are currently of great concern to regulatory agencies and consumers, particularly due to associated harmful public health implications of these residues **(Paige *et al*., 1997; Donoghue, 2003; Nisha, 2008; Darwish *et al*., 2013 and Mund *et al*., 2017)**. The most common causes of illegal antibiotic residues in foods of animal origin and poultry are failure to observe recommended withdrawal times and extra-label use of antibiotics regarding doses and/or routes of administration **(Van Dresser and Wilcke, 1989; Donoghue, 2003 and Doyle, 2006)**.

There are two major areas of concern over the presence of residues of antibiotics in animal-derived foodstuffs with regard to human health consequences. The first is direct toxic and pathological effects in human and the second is the adverse effects of these residues on human normal intestinal microflora **(Mitchell *et al*., 1998 and Nisha, 2008).**

Antibiotic residues in foods of animal origin can induce significant health problems to consumers that include hypersensitivity reactions, organ toxicity, blood dyscrasias, carcinogenicity, genotoxicity **(Nisha, 2008; Darwish *et al*., 2013 and Baynes *et al*., 2016)**.

Moreover, antibiotic residues in food create a selective pressure for the emergence and dissemination of antibiotic-resistant bacterial strains in human gut **(Silley, 2007 and Francino, 2016)**. Development of antibiotic resistant bacteria is now a serious therapeutic problem in human, which cause failure of antibiotic therapy in clinical cases **(Laxminarayan *et al*., 2013; WHO, 2015 and O'Neill, 2016).**

Fluoroquinolones are a relatively new class of synthetic, broad-spectrum antibacterials. Their popularity in clinical situations is increasing because they have several favorable properties including broad spectrum of activity against a wide range of bacteria, excellent bioavailability when given orally, good tissue penetration and a relative low incidence of adverse and toxic effects **(Sharma *et al*., 2009 and Soni, 2012)**.

Enrofloxacin was the first fluoroquinolone developed and used exclusively in veterinary medicine. It is indicated in poultry for the treatment of respiratory and intestinal tract infections especially chronic respiratory disease in broiler chickens **(Sarkozy, 2001 and**

Trouchon and Lefebvre, 2016). Ciprofloxacin is the most successful and widely used fluoroquinolone in human medicine therapeutics. It is a very potent antibacterial and because of its breadth and intense activity against most gram-negative bacteria and mycoplasma; it was also proposed for veterinary medicine use **(Nouws *et al*., 1988)**. Currently, both enrofloxacin and ciprofloxacin are the most widely used fluoroquinolones in the treatment of respiratory and intestinal tract infections of broiler chickens **(Amjad *et al*., 2005; Martinez *et al*., 2006 and Hasanen *et al*., 2016)**.

To ensure food safety and in order to protect public health from harmful adverse effects of antibiotic residues, many national regulatory authorities and international committees worldwide have established safe maximum residue limits (MRLs) or tolerances for all antibiotics allowed for use in food-producing animals. A residue below tolerance or MRL is considered safe when food at that level is consumed daily for a lifetime **(ECCR, 1990; CVMP, 2002 and CAC, 2017)**.

However, to ensure compliance with these regulations, accurate, sensitive and specific analytical methods are necessary. Many analytical methods have been developed for determination of enrofloxacin and ciprofloxacin residues in animal-derived foods. Among these methods, high-performance liquid chromatography (HPLC) with ultraviolet, fluorescence and mass spectrometry detectors are the most frequently employed techniques **(Naeem *et al*., 2006; Chang *et al*., 2008; Pena *et al*., 2010 and Hasanen *et al*., 2016)**.

Analytical methods based on ultra- performance liquid chromatography (UPLC) technique coupled to mass spectrometry (LC/MS/MS) are the techniques of choice for quantitative and confirmatory. Studies of antibiotic residues in animal-derived foods, because these methods of high selectivity and particularly high sensitivity and give sufficient data to confirm the identity of a residue **(European Commission Decision, 2002; Chang *et al*., 2010; Jang *et al*., 2013 and De Assis, 2016)**.

Therefore, the objective of the present study was to investigate the presence of the residues of enrofloxacin and ciprofloxacin in breast muscles, liver and kidneys samples of fresh broiler chicken carcasses by using an ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) method.

MATERIAL AND METHODS

Materials:

1. Samples.

A grand 90 fresh broiler chicken samples (30 each of breast meat muscles, liver and kidney samples) were randomly collected from different broiler chicken slaughterhouses and various local markets in Damietta governorate. The collected samples were kept frozen at -18 ºC until time of analysis.

2. Reagents and chemicals:

−Antibiotic standards enrofloxacin and ciprofloxacin were purchased from Dr. Ehrenstorfer GmbH (Germany).

−MS and HPLC-grade methanol (MeOH), acetonitrile (ACN) and formic acid was purchased from Fischer Company (USA).

−Solid phase extraction (SPE), cartridges (HLB) were from Waters Company (USA).

−Water was obtained through distillation and passage through a Milli-Q system (Millipore, Bedford, MA, USA).

I- Methods:

II.1. Preparation of standard solutions **(Ferrari *et al*., 2015).**

Stock standard solutions (5 mg mL^1) were prepared by dissolving enrofloxacin and ciprofloxacin in acetonitrile: water (50:50 v/v) with 0.1% formic acid. The working standard solution was prepared by dilution of stock standard solutions with acetonitrile and the antibiotics were contained in this step at variable concentrations according to their limits of quantification (LOQ) and maximum residue limit (MRL). Tuning solutions for experimental work to identify the precursors and transition products of each compound was fixed at (500 ng mL^1) and freshly prepared in ACN containing 0.1% formic acid.

II.2. Extraction and cleanup of antibiotic residues **(Ferrari *et al*., 2015).**

The samples were extracted in the regional Damietta sea port-food inspection laboratory.

II.2.1: Extraction:

−Ten gram of each of the samples of broiler chicken breast muscles, liver and 5 gram of kidney samples were homogenized in a homogenizer not more than 20 second to prepare homogenous sample for subsequent analysis steps.

–Muscles tissue (0.5 g) and kidney or liver (0.3 g) tissue of each of the samples were accurately weighed and placed in a 2 mL plastic microtube.

–Next, 1000 uL of methanol with 0.1% formic acid was added to the sample followed by shaking for 10 min at 1000g.

–The mixture was then centrifuged at 18 000g for 5 min and the upper organic phase was transferred to a 15 mL falcon tube. The residue was washed twice with 800 uL of methanol with 0.1% formic acid.

–After collection of the extracts, twelve mL of water was added to the recovered organic extract of all types of samples (muscles, kidney and liver).

II.2.2. Clean-up:

– Clean-up procedures were started with the activation of the solid phase extraction (SPE) cartridges by 2 mL of ACN followed by 2 mL of water.

–Each sample extract of each matrix was passed individually through its own (SPE) cartridge and was left until completely drained from the barrel of the cartridge.

–The samples were cleaned with 2 mL of water and 3 mL of hexane.

–For elution of enrofloxacin and ciprofloxacin from (SPE) cartridge, 5 mL of mobile phase. Then, one mL of the SPE resulting extract was placed in brown glass and used for injection into the LC-MS/MS system.

If a sample was suspected to be positive (at a low concentration), the resulted cleaned up solution could be concentrated by evaporation under gentle stream of nitrogen.

II- Method performance was conducted as mentioned by **Ferrari *et al.*, 2015** following the EU validation criteria **(European Commission Decision 2002/657/EC):**

1. Calibration curves in pure solvent were constructed for all compounds by plotting the peak area against the concentration of the seven corresponding calibration standards (4.7 to 200 ng/mL for muscles and 4.7 to 300 ng/ML for liver and kidney). In addition, calibration curves using each matrix extract were conducted to avoid matrix extract effect and to calculate the concentration of each analyte in positive samples.

2. Instrumental LODs were calculated as 3.3 times the standard deviation (SD) of the peak area of the analyte in the five replicates of the lowest concentration standard solution for each compound divided by the slope of the calibration curve. LOQs were calculated as 10 times the SD divided by the slope.

3. Meanwhile the selectivity and specificity was assessed by analyzing 10 blank samples from each matrix. The absence of background peaks, above a signal-to-noise ratio of three, at

the retention times of the target compounds showed that the method is free of endogenous interferences.

4. Identification and confirmation of the analytes were carried out by retention times, identification points of each analyte and ion ratio of selected MRM transitions. For each compound, the MRM transition with the highest intensity was used for quantification (quantifier ion), while the other transition was used for confirmation (qualifier ion).

III- Determination and quantification of antibiotic residues was conducted according to **Han *et al*., (2015)**.

The chromatographic analyses were performed at toxicology, drugs and hormones residues Lab, MEWA, Saudi Arabia, on an Acquity UPLC system, and separations were achieved using an Acquity UPLC BEH C18 column (1.7 μm particle size, 100 mm × 2.1 mm; Waters). The analytes were separated with a mobile phase consisting of acetonitrile (eluent A) and 0.1% formic acid in water (eluent B) at a flow rate of 0.3 mL $min^{-}1$. The separation was performed at 40°C, applying the following gradient program: 0-0.5 min, 5% A; 0.5-1 min, linear increase to 10% A; 1-3 min, linear increase to 40% A; 3-4 min, linear increase to 90% A; 4-4.1 min, decrease to 40% A; and finally, 4.1-6.5 min, 5% A. The samples were kept in an autosampler at 15 °C.

The mass spectrometry analyses were carried out using a Waters Acquity UPLC H class that was accompanied with triple-quadrupole mass spectrometry equipped with an electrospray ion source.

The instrument was operated using an electrospray (ESI) source in positive mode with the following parameters: 0.5 kV capillary voltage, 30 V cone voltage, 500 C desolvation temperature, and 1000 L h1 desolvation gas (nitrogen>99.999%) flow. **Young and Tran (2014)** performed data acquisition using MassLynx V 4.1 software with the Quanlynx program (Waters) where the antibiotics tuning protocol was as described**.**

RESULTS AND DISCUSION

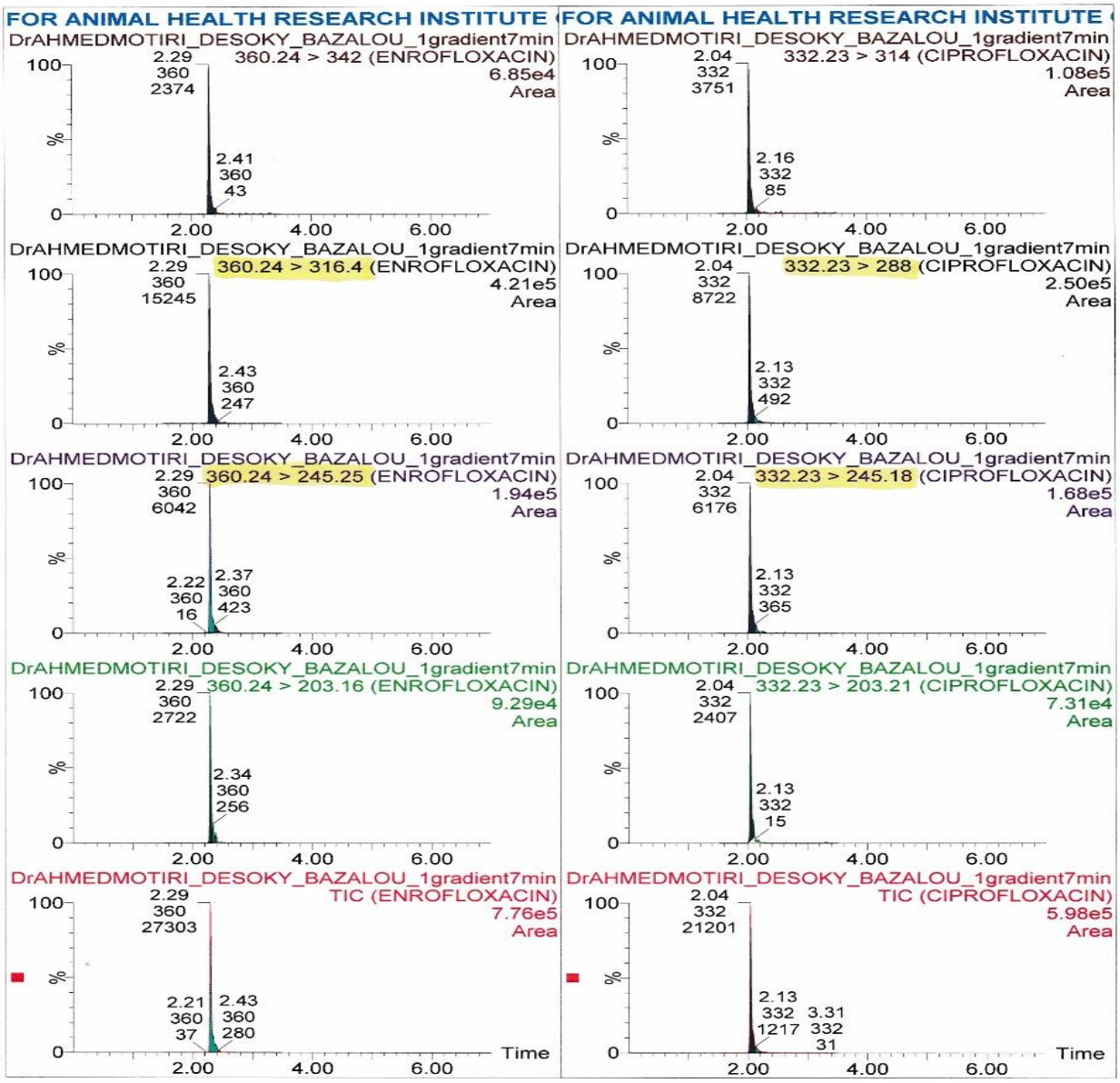

Fig. (1): Representative chromatogram showing total ion chromatograph (TIC) and daughter ions related to enrofloxacin and ciprofloxacin as generated by the Quanpedia software and confirmed by the experimental work.

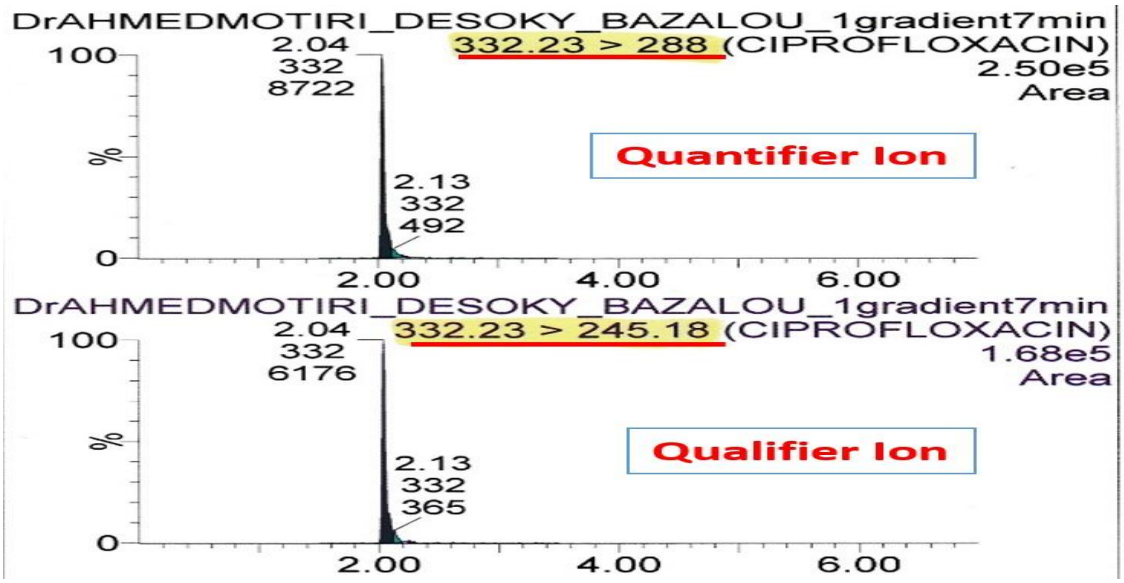

Fig. (2): Representative chromatogram showing quantifier and qualifier ions of ciprofloxacin as generated by the Quanpedia software and confirmed by the experimental work.

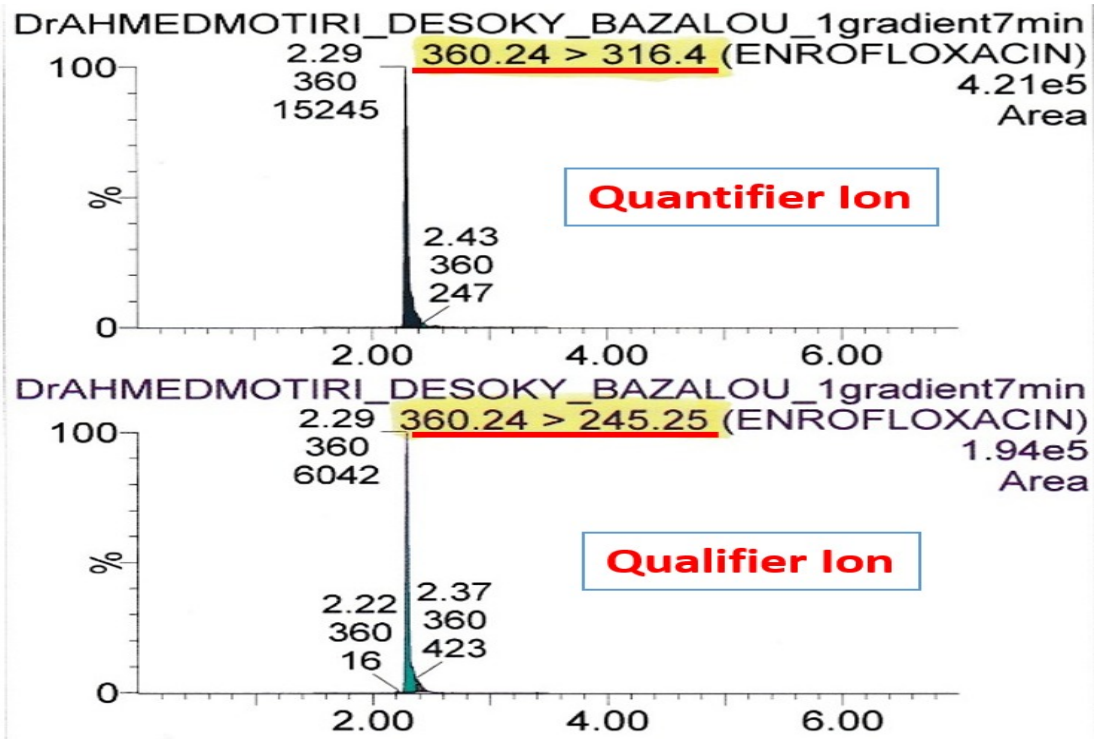

Fig. (3): Representative chromatogram showing quantifier and qualifier ions of enrofloxacin as generated by the Quanpedia software and confirmed by the experimental work.

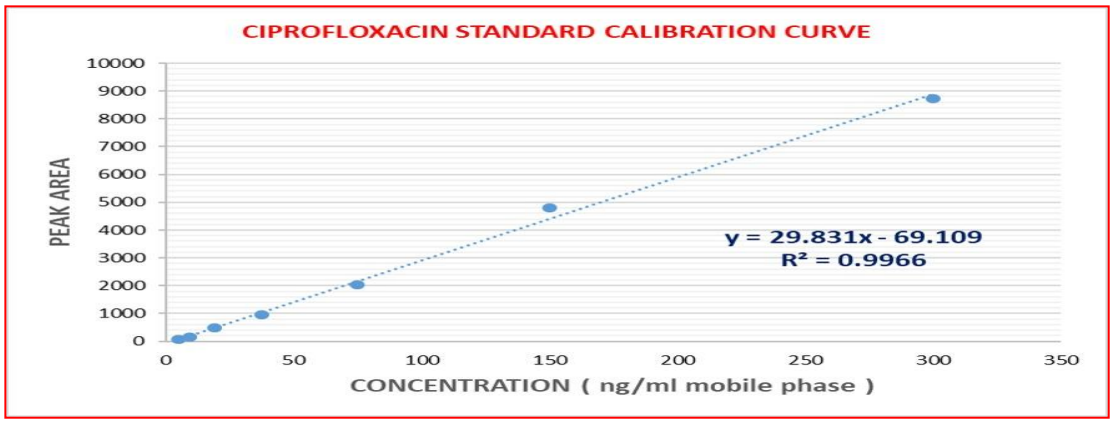

Fig. (4): Matrix matched standard calibration curve of ciprofloxacin for kidney extract dissolved in mobile phase (Coefficient of Determination, R2=0.9966).

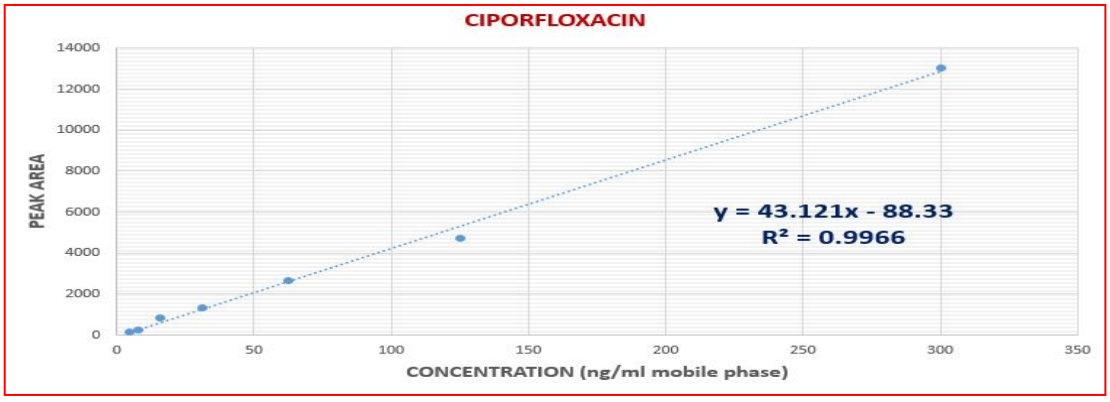

Fig. (5): Matrix matched standard calibration curve of ciprofloxacin for liver extract dissolved in mobile phase (Coefficient of Determination, R2=0.9966).

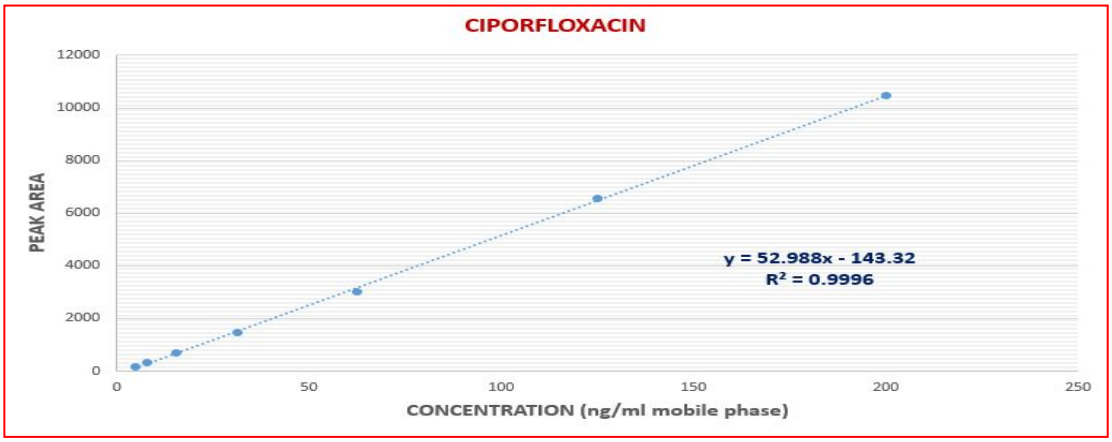

Fig. (6): Matrix matched standard calibration curve of ciprofloxacin for muscles extract dissolved in mobile phase (Coefficient of Determination, R2=0.9996).

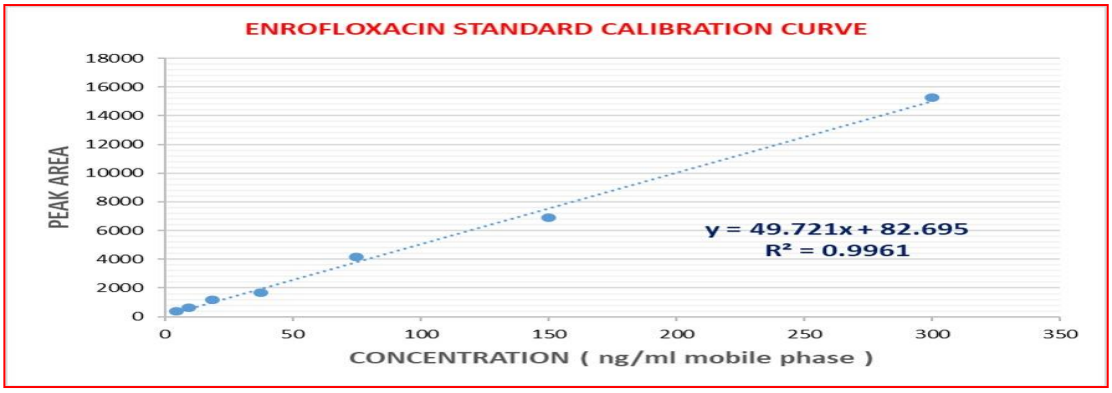

Fig. (7): Matrix matched standard calibration curve of enrofloxacin for kidney matrix dissolved in mobile phase (Coefficient of Determination, R2=0.9961).

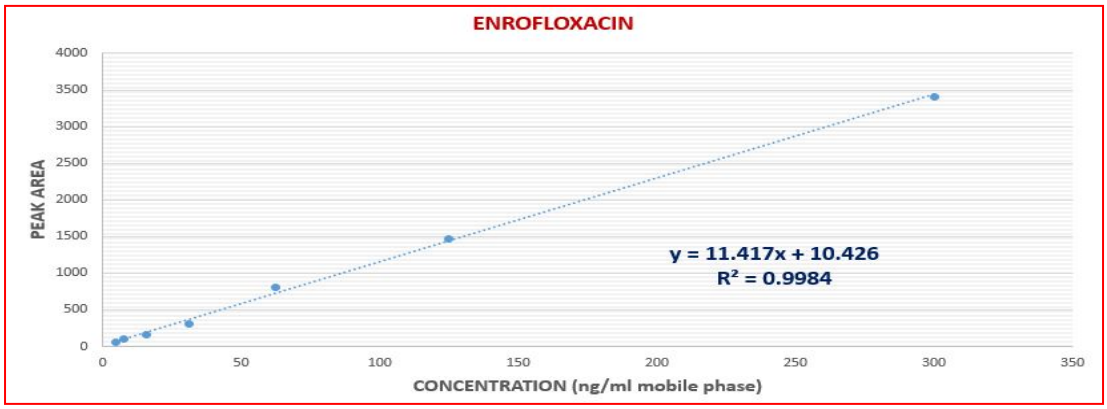

Fig. (8): Matrix matched standard calibration curve of enrofloxacin for liver matrix dissolved in mobile phase (Coefficient of Determination, R2=0.9984).

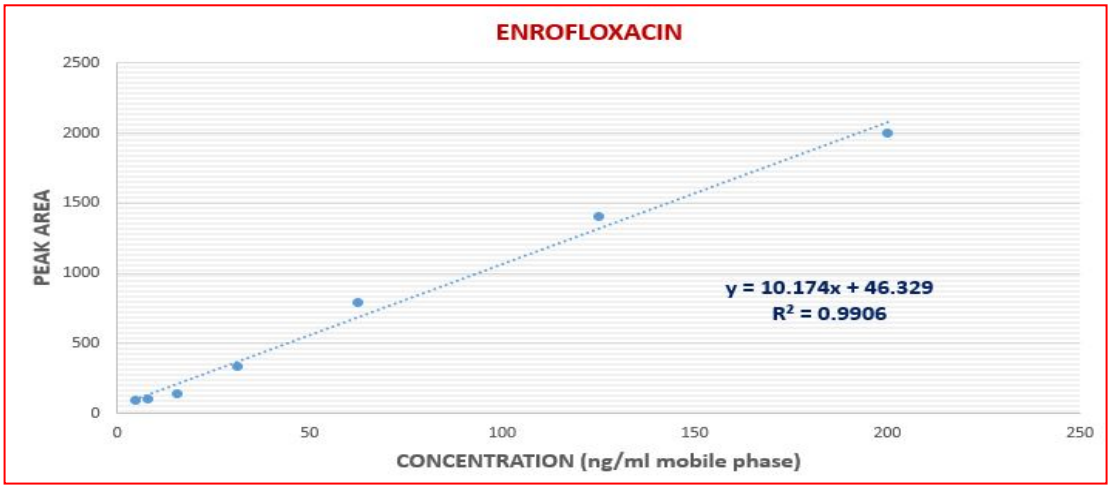

Fig. (9): Matrix matched standard calibration curve of enrofloxacin for muscles matrix dissolved in mobile phase (Coefficient of Determination, R2=0.9906).

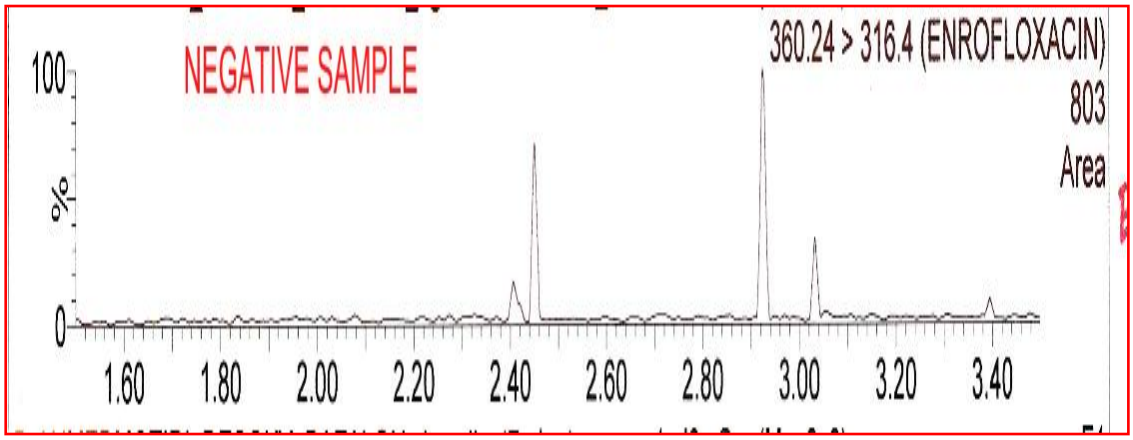

Fig. (10): Chromatograph of negative sample against enrofloxacin.

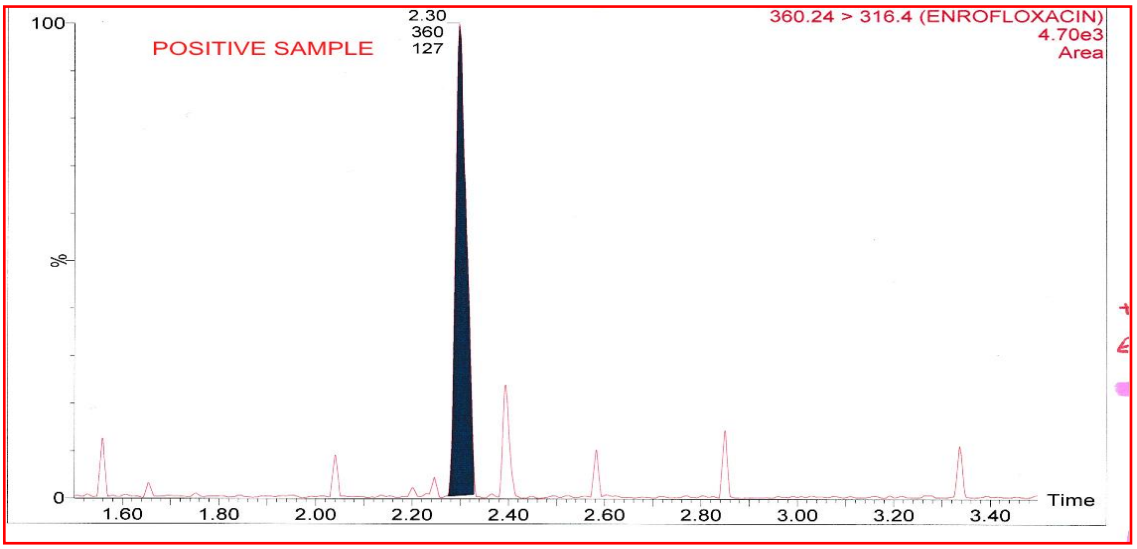

Fig. (11): Chromatograph of enrofloxacin positive muscles sample (8.11 ng/g).

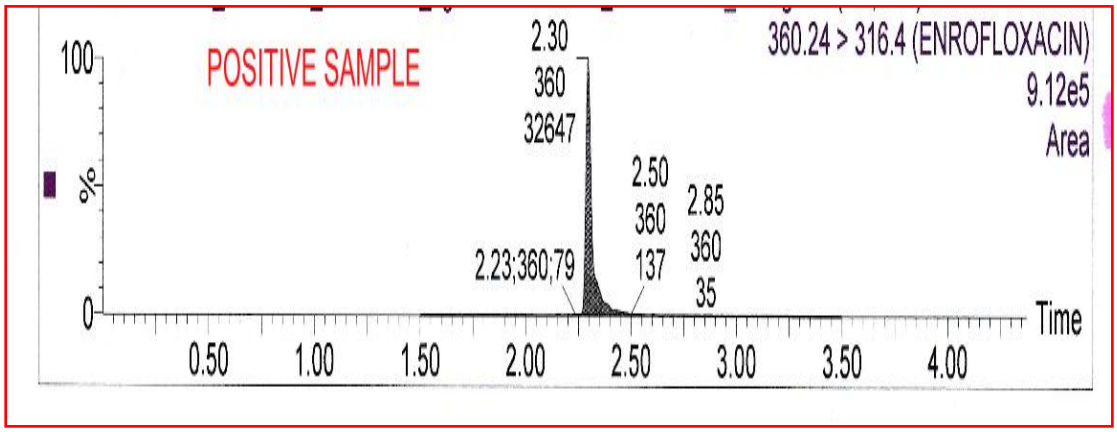

Fig. (12): Chromatograph of enrofloxacin positive kidney sample (579.6 ng/g).

Table (1): Limit of detection, limit of quantification (ng/g) and retention time of enrofloxacin and ciprofloxacin in muscles, liver and kidney of broiler chicken.

Antibiotics	Limit of detection (LOD)* (ng/g)			Limit of quantification (LOQ)* (ng/g)			Retention time
	muscles	liver	kidney	muscles	liver	kidney	
Ciprofloxacin	0.81	1.00	1.10	2.41	3.00	3.21	2.04
Enrofloxacin	1.43	1.47	1.30	4.41	4.30	3.95	2.29
*According to **Shrivastava & Gupta (2011)**.							

Table (2): Fluoroquinolones residue in the examined samples and the judgment in accordance to maximum residue limit (n=30 each).

Samples type	Antibiotic	Positive samples		Residues concentration (µg/kg)	Legal status in accordance with maximum residue limit (MRL)*	Judgment
		No.	%			
Breast muscles	Ciprofloxacin	-	-	-	Compliant	Accepted
	Enrofloxacin	1	3.33	8.11	Compliant	Accepted
Liver	Ciprofloxacin	-	-	-	Compliant	Accepted
	Enrofloxacin	-	-	-	Compliant	Accepted
Kidney	Ciprofloxacin	-	-	-	Compliant	Accepted
	Enrofloxacin	1	3.33	579.6	Non-compliant	Rejected
***Maximum residue limit (MRL) (100, 200 and 300 µg/kg for muscles, liver and kidney respectively) according to Commission Regulation EU/No. 37 (2010).**						

Public health agencies in many countries rely on detection of antibiotic residues by mass spectrometry that, being a specific detector, affords unambiguous confirmation of contaminants in foodstuff. **European Commission Decision 657/EC (2002)** states that, the "Methods based only on chromatographic analysis without the uses of molecular spectrometric detection are not suitable for use as confirmatory methods".

Determination of the optimal multiple reaction monitoring (MRM) transitions for both quantifier and a qualifier ion was carried out by infusing the individual standard at concentration level around 1 µg/mL. The quantifier ion was chosen as the most abundant product ion and the qualifier ion was chosen as the second-most abundant product ion **(Zumwalt and Moore, 2006)**.

Fig. 1, 2 and 3 showed total ion chromatography (TIC) and representative chromatograms of quantifier and qualifier ions of each ciprofloxacin and enrofloxacin as generated by the Quanpedia™ software Waters®. The results were confirmed through the experimental work. Meanwhile, the most abundant fragment was selected as the quantifier ion. The second most abundant fragment was selected as the qualifier ion **(Ferrari *et al*, 2015) and (Zumwalt and Moore, 2006)**.It was found that ciprofloxacin had quantifier and qualifier ions (m/z) at positive ionization mode of 288 and 245.18 respectively, meanwhile enrofloxacin had quantifier and qualifier ions of 316.4 and 245.25 respectively Fig. (2, 3).

The calibration curves were linear in the range of concentrations assessed with a correlation coefficient more than 0.99 for both antibiotics Fig. (4, 5, 6, 7, 8 and 9).

According to methods for the determination of limit of detection and limit of quantitation cited by **Shrivastava and Gupta (2011)**, (Table 1) showed the limits of detection of enrofloxacin and ciprofloxacin in different examined tissues, the lowest value was recorded for the detection limit of ciprofloxacin in muscles and the highest level was for enrofloxacin in liver. It was observed that, the method is free of endogenous interferences that may interfere with the analyte identification and quantification. This fact was supported by the absence of background peaks at the retention times of the target compounds when analyzing 10 blank samples of each matrix (muscles, liver and kidney). Fig.(10) is a representative chromatogram of negative sample that had no background peaks at the retention time (2.29 min) specified for enrofloxacin.

Fig.(11,12) showed representative chromatograms of positive enrofloxacin samples.

The first positive sample was for breast muscles and recorded 8.11 µg/kg by 3.33% indicating that it still under the maximum residue limit recommended for enrofloxacin in muscles (100 µg/kg) **Commission Regulation EU/No. 37 (2010)** so, it was a compliant sample and safe for human consumption. Meanwhile, the second positive sample (a kidney sample) recorded a concentration of 579.6 µg/kg by 3.33% indicating that it contained enrofloxacin residue higher than the recommended level in kidney (300 µg/kg) **Commission Regulation EU/No. 37 (2010)** so, it was non-compliant and not safe for human consumption as showed in (Table 2). This result demonstrated that enrofloxacin was used in broiler chicken farms and recommended withdrawal times of this antibiotic was not properly observed. **Jelena *et al*., (2006) and De Assis *et al*.,(2016)** reported that, the presence of enrofloxacin residues at concentrations higher than the drugs' maximum residue limit (MRL) was found only during the treatment period and after four days of withdrawal, the levels were lower than MRL. Therefore, the obtained results confirmed misuse of antibiotics especially enrofloxacin in broiler chicken farms. Broiler chickens are marketed and slaughtered during dosing period or proper withdrawal time of this antibiotic has not been followed.

Results of our study are lower than those reported by **Rokni *et al*., (2007).** They analyzed in 270 broiler chickens muscle, liver and kidney samples and mentioned that, the samples (100.0%) are contained enrofloxacin residues. Whereas, **Attari *et al*., (2014)** examined ninety broiler chickens carcasses found that 82 (91.1%) contained the residues of enrofloxacin. Moreover, **Panzenhagen *et al*., (2016)** reported that 22.2% of the analyzed broiler chicken samples contained enrofloxacin residues detected by LC-MS/MS. On contrary, **Weiss *et al*., (2007)** analyzed in 299 samples of broiler chicken meat in Italy and could not detect enrofloxacin antibiotic residues.

On other hand, results showed that none of the examined samples had residues of ciprofloxacin. This may be due to ciprofloxacin is not commonly used in the treatment of broilers chickens in Damietta governorate farms. This could be attributed to the fact that ciprofloxacin is approved in human medicine and not in veterinary medicine and there are no many ciprofloxacin veterinary pharmaceutical formulations available in the market.

CONCLUSIONS AND RECOMMENDATIONS

In conclusion, the present study confirmed misuse of enrofloxacin in broiler farms and lack of implementation of recommended withdrawal times. Moreover, the present study stresses the

need for stricter regulation for the use of antimicrobials in the poultry industry as well as the inspection of broiler chickens for antibiotic residues prior to marketing and slaughtering.

There is no a routine screening program for slaughtered animal is practiced in Egypt. So, establishment of effective antibiotic-residue-monitoring system is necessary to ensure that antibacterial agents are not present at levels that may possess risks to the public health and proper withdrawal times before marketing and slaughtering have been followed and this should be applied to safeguard public health against adverse health effects of antibiotic residues.

National authorities should also adopt more judicious approaches to ensure prudent use of antibiotics in broiler chickens. Poultry producers need to be aware to best poultry practices to reduce infections and consequently reduce the use of antibiotics.

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بقايا بعض الفلوروكوينولون في صدور وأكباد وكُلى الدجاج اللاحم الطازج

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المُلخص

تشكل بقايا المضادات الحيوية في الأطعمة ذات الأصل الحيواني مصدر قلق كبير للوكالات التنظيمية والمستهلكين، ولا سيما بسبب الآثار الصحية الضارة المرتبطة بهذه البقايا, هناك مجالان رئيسيان للقلق بشأن وجود بقايا من المضادات الحيوية في المواد الغذائية ذات الأصل الحيواني فيما يتعلق بصحة الإنسان, الأول هو التأثيرات السامة والمرضية المباشرة, والثاني هو الآثار الضارة لهذه البقايا على البكتيريا المعوية الطبيعية للإنسان, الفلوروكينولونات وهي مضادة للبكتيريا مستخدمة بشكل شائع في مزارع انتاج الدجاج اللاحم لعلاج التهابات الجهاز التنفسي والأمعاء, ولقد أجريت هذه الدراسة للتحقق من وجود بقايا الفلوروكينولونات الأكثر استخدامًا وهي enrofloxacin و ciprofloxacin في عينات عضلات الصدر وأكباد وكُلى ذبائح دجاج التسمين الطازج المباع بالأسواق وذلك باستخدام تقنية UPLC-MS/MS, وأوضحت النتائج المتحصل عليها أن عدد 2 عينة كانت إيجابية لتحليل بقايا المضادات الحيوية من مضاد الإنروفلوكساسين, كانت الأولى عينة إيجابية لعضلات الصدر وسجلت 8,11 ميكروجرام/كيلو جرام وكانت في الحد المسموح به, وكانت العينة الإيجابية الثانية هي عينة كُلى سجلت تركيزًا وقدره 579,6 ميكروجرام/كيلو جرام أعلى من الحد المسموح به, ومن ناحية أخرى أظهرت النتائج أن جميع العينات التي خضعت للفحص لم يكن بها بقايا سيبروفلوكساسين, وقد تم مناقشة النتائج والمخاطر الصحية الناجمة عن وجود بقايا المضادات الحيوية مع وضع مقترح بالتوصيات اللازمة.

الكلمات الدالة:

المضادات الحيوية - الفلوروكوينولون - الدجاج اللاحم - مخلفات الذبح القابلة للأكل - الكروماتوجراف السائل فائق الأداء UPLC.