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DETERMINATION OF RESIDUES OF FLUOROQUINOLONES IN POULTRY MEAT BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

Rajeev Sharma * and Reena S Lawrence

Department of Chemistry, Sam Higginbottom University of Agriculture, Technology & Sciences, Prayagraj Allahabad - 211007, Uttar Pradesh, India.

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Correspondence to Author:
Mr. Rajeev Sharma

Department of Chemistry,
Sam Higginbottom University of Agriculture, Technology & Sciences, Prayagraj Allahabad - 211007, Uttar Pradesh, India.

E-mail: sharmachem1979@gmail.com

ABSTRACT: Three analytical methods were developed for the determination of selected fluoroquinolones in chicken meat by high performance liquid chromatographic technique. In each method, we took two fluoroquinolones (Danofloxacin and difloxacin; Ciprofloxacin and enrofloxacin; Ofloxacin and pefloxacin) research study. Rapid, simple and sensitive sample treatment procedures were developed and used to extract the fluoroquinolones from the chicken tissues with good recoveries. Extraction recovery from chicken tissues was in the range of 86.96 to 97.49 %, depending upon the analytes. The shape of the curves for all drugs was linear in the investigation concentration range 10-1000 ng/ml, and the correlation coefficient of the linearity curves for all drugs was in the range of 0.9707- 0.9991. The detection limits for fluoroquinolones in chicken tissues were well below the Maximum Residual Limits (MRLs) for antibiotics. The limit of detection (LOD) was in the range of 9-12 µg/kg, and the limit of quantification (LOQ) was in the range of 30-40 µg/kg for all analytes in chicken tissues. The separation results and parameters of the methods were evaluated from spiked tissue samples, and results were found good and within range. The proposed methods can be applied for routine determination of these fluoroquinolones in chicken tissue samples.

INTRODUCTION: Fluoroquinolones are an important family of synthetic antibacterials used in both human and veterinary medicine. In the veterinary field, they are used for the prophylaxis and treatment of veterinary diseases in most types of farm animals [1]. Their common skeleton is 4-oxo-

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These fluoroquinolones 1, 4-dihydroquinoline, where the pharmacological unit consists of a pyridine ring with a carboxyl group, a piperazinyl group, and a fluorine atom placed at position [3, 7] and [6], respectively [2]. Drugs belonging to the fluoroquinolones class such as danofloxacin, difloxacin ciprofloxacin, enrofloxacin, ofloxacin, and pefloxacin are used in veterinary practice to control various diseases and to lead to the poultry production of healthy birds.

These fluoroquinolones are used in the treatment of systemic infections, including respiratory tract, gastrointestinal tract, urinary tract, and dermal

infections. In the veterinary field, they are used not only for the treatment and prevention of many diseases but also AS feed additives to increase the body mass. Drugs and their metabolites remain within the body for a longer time after their administration. The concentrations of these residues differ significantly from tissue to tissue and are usually detected higher in organs that metabolize and secrete them efficiently, such as the liver, kidneys, or storage tissues like body fat. Drug resistance is caused by residues of these drugs [3]. Drug residues in foods have been said to cause allergic reactions, toxicity, technological problems in fermented products, and the development of antibiotic resistance in human pathogens. It has been documented that a major route of transmission of resistant micro-organisms from animals to humans is through the food chain [4,] 5. Residues of Fluoroquinolones may occur in animal tissues if the adequate withdrawal times are not observed or if the compounds are improperly administered [6].

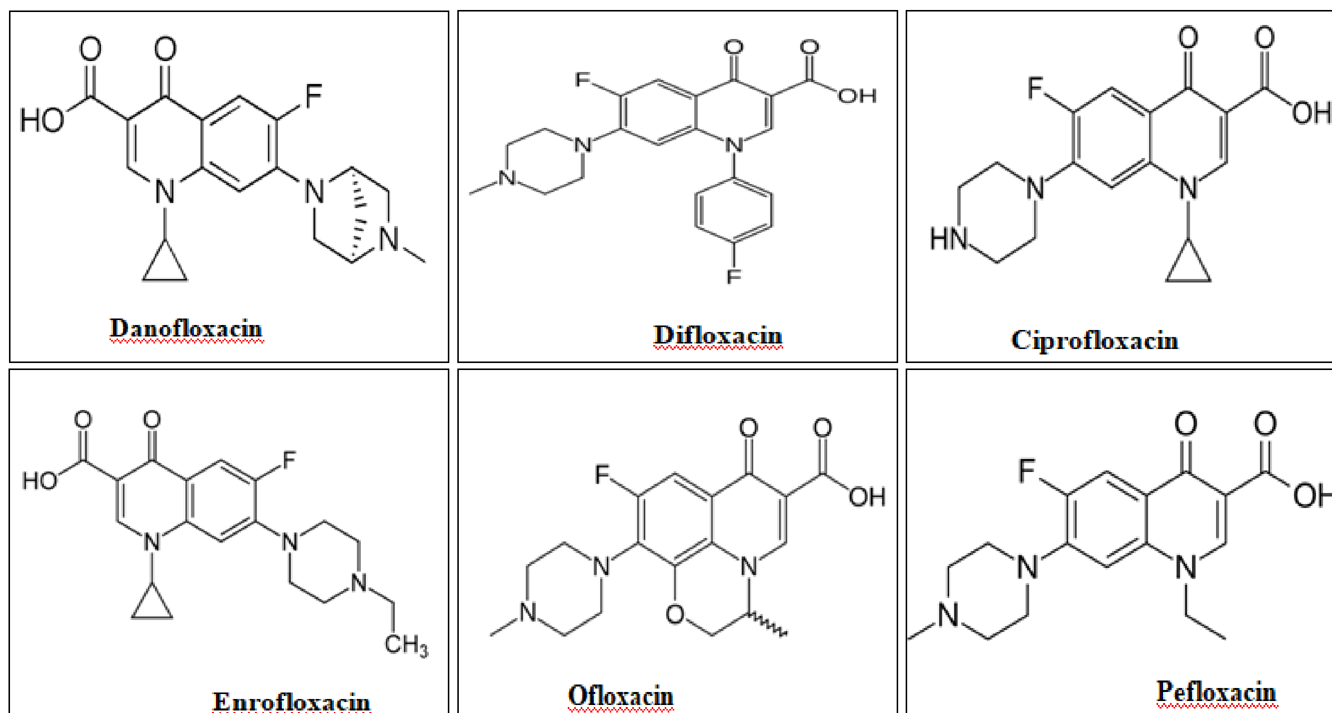

FIG. 1: CHEMICAL STRUCTURE OF FLUOROQUINOLONES

To ensure safety in human foodstuffs, the European Union (EU) has taken steps such as setting tolerance levels for fluoroquinolones in products of animal origin. According to European Union, the Maximum Residues Limits (MRLs) are 100 µg/kg for the sum of enrofloxacin and ciprofloxacin as well as 200 µg/kg for danofloxacin and 300 µg/kg for difloxacin for chicken muscle [7]. There are no any Maximum Residues Limits (MRLs) according to International Regulatory Agencies (European Union and Codex Alimentarius Commission) for ofloxacin and pefloxacin for chicken muscle. This has raised the need to develop sensitive methods for measuring the residual amount of these drugs to facilitate quality control analyses of food products. Various analytical techniques have been reported for the estimation of drugs in different animal products. High-performance liquid chromatography (HPLC) has been widely used to determine trace elements in drug analysis based on its high selectivity and sensitivity. HPLC is used to separate, identify and quantify different chemical components. It has been successfully applied to the detection of drug residues in foods of animal origin [8]. Many studies have been published concerning the analysis of fluoroquinolones in various biological matrices and only a few reports have described the determination of fluoroquinolones in chicken muscle. High-performance liquid chromatography (HPLC) with fluorescence [9, 19], ultraviolet [20], or mass spectrometric [21] detection methods has been established to determine these fluoroquinolones in chicken muscle. All the above-mentioned published methods involved HPLC's use to determine fluoroquinolone residues in chicken muscle. Most of them were using HPLC coupled with

fluorescence detector and only few reports have reported HPLC with UV detector technique for the estimation of fluoroquinolones in chicken muscle. Photodiode array detector was selected for the determination of these fluoroquinolones in chicken muscle as the advanced optical detection, high chromatographic sensitivity, and full scanning of uv/visible range. Most of these methods were generally based on solvent phase extraction techniques for the extraction of fluoroquinolones from biological samples. These methods involved time-consuming and complicated sample treatment procedures and required large amounts of organic solvents and other chemicals. To avoid these problems, improved sample pretreatment procedures presented good recoveries.

We recently reported the HPLC methods for the determination of fluoroquinolones in the poultry meat with simple and fast sample preparation procedures [22, 23]. The achieved analytical parameters showed good results in these methods. We have focused on the analysis of danofloxacin, difloxacin, ciprofloxacin, enrofloxacin, ofloxacin, and pefloxacin used in the poultry industry to control various diseases, and three different methods have been developed for the determination of these fluoroquinolones in chicken muscle by high-performance liquid chromatography. This paper reports the development of extraction procedures with good analytical results and liquid chromatographic methods for the determination of fluoroquinolones in chicken muscle.

MATERIALS AND METHODS:

Extraction of Samples: The chicken muscle samples were obtained from the healthy birds that were not treated with any veterinary drugs. The tissue samples were deep-frozen until analysis for determination of drug residues. The tissue samples were thawed to room temperature and then cut into small pieces.

Extraction Procedure for Danofloxacin and Difloxacin: An accurately weighed 2.0 gm of tissues sample was placed in 15.0 ml polypropylene centrifuge tube and homogenized using a tissue homogenizer with 4.0 ml of milli-q-water. From this resultant homogenate, 2.0 ml was taken into a 15.0 ml polypropylene centrifuge tube, and to add 1.0 ml of amine buffer. The mixture was then sonicated and left undisturbed for 15 min. After that, 2.0 ml of acetonitrile was added to it, and the tube was tightly capped and vortexed for 5 minutes using Spinix vortex. The mixture was centrifuged for 30 min at 4000 rpm using Research Centrifuge R-24. The supernatant was decanted into another tube and centrifuged once again at 8,000 rpm for 30 min using Cooling centrifuge CM-12. Finally, the supernatant was filtered using 0.2µm (nylon + prefilter) mdi syringe filter (Advanced Microdevices Pvt. Ltd., India). 20 µl of this filtrate was then injected into the HPLC system for analysis.

Extraction Procedure for Ciprofloxacin and Enrofloxacin: An accurately weighed 1.0 gm of tissues sample was placed in 15.0 ml polypropylene centrifuge tube and homogenized using tissue homogenizer with 6.0 ml of phosphate buffer. From this resultant homogenate, 3.0 ml was taken into a 15.0 ml polypropylene centrifuge tube and to this added 2.0 ml of acetonitrile and the tube was tightly capped and vortexed for 5 min using Spinix vortex. The mixture was then sonicated and left undisturbed for 10 min. Later, the mixture was centrifuged for 15 min at 5000 rpm using Research Centrifuge R-24. The supernatant was decanted into another tube and centrifuged once again at 8,000 rpm for 30 min using Cooling centrifuge CM-12. Finally, the supernatant was filtered using 0.2µm (nylon + glass fibre) mdi syringe filter (Advanced Microdevices Pvt. Ltd., India). 20 µl of this filtrate was then injected into the HPLC system for analysis.

Extraction Procedure for Ofloxacin and Pefloxacin: An accurately weighed 0.5 gm of tissues sample was placed in a 15.0 ml polypropylene centrifuge tube and homogenized using tissue homogenizer with 2.0 ml of amine buffer solution. After that, added 1.0 ml of potassium chloride solution. The mixture was then sonicated and left undisturbed for 15 min. After that, 2.0 ml of acetonitrile was added to it, and the tube was tightly capped and vortexed for 5 min using Spinix vortex. The mixture was centrifuged for 15 min at 5000 rpm using Research Centrifuge R-24. The supernatant was decanted into another tube and centrifuged once again at 8,000 rpm for 30 min using Cooling centrifuge CM-12. Finally, supernatant was filtered using a 0.45 µm (nylon +

prefilter) MDI syringe filter (Advanced Microdevices Pvt. Ltd., India). 20 µl of this filtrate was then injected into the HPLC system for further analysis.

Instrumentation: The High-Performance Liquid Chromatography (HPLC) was carried out on a Shimadzu system (Shimadzu, Kyoto, Japan). The system was equipped with a Quaternary gradient pump (LC-10ATvp), a Photodiode array detector (SPD-M10Avp), a Column oven (CTO-10ASvp), a System controller (SCL-10Avp), a Degasser (DGU-14 Avp), and an Autoinjector (SILL-10 ADvp). The CLASS VP Software package was used for instrument control, data acquisition, and data analysis.

A glass vacuum filtration apparatus was employed to filtrate the buffer solution using a 0.2 µm nylon membrane filter obtained from Borosil, India. Prior to use, solvents were degassed by sonication in Ultrasonic bath Rivotech (Riviera Glass Pvt. Ltd., India). Semi microbalance CPA225D (Sartorius Weighing Technology, Germany) was used to weighing reference standards, and Analytical balance Precisa XB 2220M-DR (Adair, Dutt & Co. Pvt. Ltd., India) was used to weighing tissue samples. Cyberscan pH meter (Eutech Instruments, Malaysia) was used to adjust pH of buffer solution and Tissue homogenizer Polytron PT 1600E (Kinematica AG, Switzerland) was used to homogenize tissue samples during pretreatment. A vortex mixer Spinix (Tarsons Products Pvt. Ltd., India) was used to mix tissue samples employed for the sample preparation, and a Research Centrifuge R-24 (REMI, India), as well as Cooling Microfuge CM-12 (REMI, India), were used to perform the extractions.

Chromatographic Conditions:

Conditions for Danofloxacin and Difloxacin: The isocratic mobile phase consisting of buffer (0.5% triethylamine, pH 3.5 adjusted with ortho-phosphoric acid) and acetonitrile in the ratio of (80:20, v/v) was used throughout the analysis. The flow rate of the mobile phase was maintained at 0.6 ml/min, and the injection volume was 20 µl. A photodiode array detector was operated at a wavelength of λ_{max} = 280 nm. The retention times for danofloxacin and difloxacin were about 8 and 15 min, respectively. The column was carried out at an oven temperature of 30º C and the total run time for the analysis of both drugs was 20 min. A reverse phase C18 column, Hypersil BDS, 250 mm × 4.6 mm with a particle size of 5µm (Thermo Scientific) was used as a stationary phase for separation of the compounds.

Conditions for Ciprofloxacin and Enrofloxacin: The isocratic mobile phase consisting of buffer (0.025M Potassium phosphate, pH 3.25 adjusted with ortho-phosphoric acid) and acetonitrile in the ratio of (75:25, v/v) was used throughout the analysis. The flow rate of the mobile phase was maintained at 0.5 ml/min, and the injection volume was 20 µl. A photodiode array detector was operated at a wavelength of λ_{max} = 277 nm. The retention times for ciprofloxacin and enrofloxacin were about 10 and 13 min, respectively. The column was carried out at an oven temperature of 30 ºC and the total run time for the analysis of both drugs was 20 min. A reverse phase, Luna C18 column, 250 mm×4.6 mm with a particle size of 5 µm (Phenonmenax) was used as a stationary phase for the separation of the compounds.

Conditions for Ofloxacin and Pefloxacin: The isocratic mobile phase consisting of buffer (0.025M triethylamine, pH 4.5 adjusted with ortho-phosphoric acid) and acetonitrile in the ratio of (85:15, v/v) was used throughout the analysis. The flow rate of the mobile phase was maintained at 0.750 ml/min, and the injection volume was 20 µl. A photodiode array detector was operated at a wavelength of λ_{max} = 282 nm. The retention times for ofloxacin and pefloxacin were about 7 and 10 min, respectively. The column was carried out at an oven temperature of 30 ºC and total run time for the analysis of both drugs was 20 min. A reverse phase C18 column, Purospher Star, 250 mm × 4.6 mm with a particle size of 5 µm (Merck) was used as a stationary phase for separation of the compounds.

Chemicals and Reagents: Reference standards of Danofloxacin (Batch # 7302Z), Difloxacin HCL (Batch # 6067X), Ciprofloxacin hydrochloride (Batch # BCBD1343V), Enrofloxacin (Batch # SZBA336XV), Ofloxacin (Batch # SLBB1877V), and Pefloxacin mesylate dihydrate (Batch # SLBC5834 V) were obtained from Sigma-Aldrich, USA. Acetonitrile (HPLC-grade), Methanol (HPLC-grade), Triethylamine, Potassium

dihydrogen phosphate and orthophosphoric acid were procured from Merck Specialties Pvt. Ltd., India. Water was purified by Milli-q water system (Millipore, France) and this water was used throughout analysis. Stock solutions of 1 mg/ml of danofloxacin, difloxacin, ofloxacin, pefloxacin were prepared in HPLC grade methanol. Stock solutions of 1 mg/ml of ciprofloxacin and enrofloxacin were prepared in water and diluted with HPLC grade methanol.

All stock solutions were stored refrigerated at 4 °C. Individual working solutions of drugs were prepared daily from the stock solutions by diluting with the mobile phase. The working solutions used to spike the tissue samples were prepared by mixing the individual stock solutions.

RESULTS AND DISCUSSION:

Linearity: Linearity curve was plotted concentration of the sample (x-axis) against the concerned peak (y-axis) area for drugs using chicken tissues homogenate. Nine point linearity curves were obtained in a concentration range from 10 to 1000 ng/ml for all analytes. The shape of the curves for all drugs was found to be linear in the investigation concentration range, and correlation coefficient of the linearity curves for drugs was in the range of 0.9707- 0.9991. Linearity parameters of linearity curves for drugs are shown in **Table 1.**

TABLE 1: PARAMETERS OF LINEARITY CURVES FOR DRUGS USING CHICKEN TISSUES HOMOGENATE

Analytes	Retention time (min)	Linearity range (ng/ml)	Shape	Regration equation	Correlation co-efficient
Danofloxacin	8	10-1000	Linear	y = 223.41x -1179.3	0.9972
Difloxacin	15	10-1000	Linear	y = 189.93x -271.24	0.9991
Ciprofloxacin	10	10-1000	Linear	y = 233.38x - 2357.5	0.9801
Enrofloxacin	13	10-1000	Linear	y = 279.19x - 1713.9	0.9895
Ofloxacin	7	10-1000	Linear	y = 199.66x - 564.62	0.9707
Pefloxacin	10	10-1000	Linear	y = 232.92x - 945.52	0.9928

Recovery: Recovery for drugs from chicken tissues was determined by comparing the peak areas obtained with extracted samples with those obtained with un-extracted standards. Recovery for all drugs from chicken tissues homogenate was assessed at four spike levels. Extraction recovery from chicken tissues was in the range of 86.96 to 97.49 %, depending upon the analytes. Recoveries for drugs from chicken tissues are shown in **Table 2.**

TABLE 2: RECOVERIES FOR DRUGS FROM CHICKEN TISSUES AT DIFFERENT CONCENTRATION LEVELS

Theoretical spiked conc (ng/ml)	Name of the analyte	Experimentally detected conc (ng/ml)	Recovery from matrix (%)	Name of the analyte	Experimentally detected conc (ng/ml)	Recovery from matrix (%)
50	Danofloxa	46.90	93.79	Difloxacin	48.74	97.48
100	cin	94.57	94.57		93.08	93.08
250		227.73	91.09		243.73	97.49
500		462.75	92.55		467.65	93.53
50	Ciprofloxa	45.68	91.35	Enrofloxa	45.80	91.60
100	cin	91.46	91.46	cin	92.23	92.23
150		131.88	87.92		140.76	93.84
200		183.58	91.79		183.06	91.53
50	Ofloxacin	45.27	90.53	Pefloxacin	48.28	96.55
100		86.96	86.96		96.89	96.89
250		226.70	90.68		234.20	93.68
500		448.20	89.64		472.30	94.46

Detection Limits: Limit of detection (LOD) and limit of quantification (LOQ) were determined by tissues homogenate spiking with serial dilutions of analytes and observed at various concentration levels in a concentration range from 10 to 100 ng/ml. The analysis of samples evaluated limit of detection and limit of quantification with the known concentration of drugs and by establishing the minimum level at which the analyte can be reliably detected or quantified. The limit of detection (LOD) was in the range of 9-12 µg/kg and the limit of quantification (LOQ) was in the

range of 30-40 µg/kg for all analytes in chicken tissues which are well below the Maximum Residue limits (MRLs). Detection limits for drugs in chicken tissues are shown in **Table 3.**

Representative chromatograms for a mixture of both drug standards and spiked chicken tissues homogenate are shown in **Fig. 2.**

TABLE 3: DETECTION LIMITS (LOD AND LOQ) FOR DRUGS IN CHICKEN TISSUES

Analytes	Biometric	Retention time (min)	Detection range (ng/ml)	LOD (µg/Kg)	LOQ (µg/Kg)
Danofloxacin	Chicken muscle	8	10-100	10.50	35
Difloxacin	Chicken muscle	15	10-100	12.00	40
Ciprofloxacin	Chicken muscle	10	10-100	10.50	35
Enrofloxacin	Chicken muscle	13	10-100	9.00	30
Ofloxacin	Chicken muscle	7	10-100	10.50	35
Pefloxacin	Chicken muscle	10	10-100	9.00	30

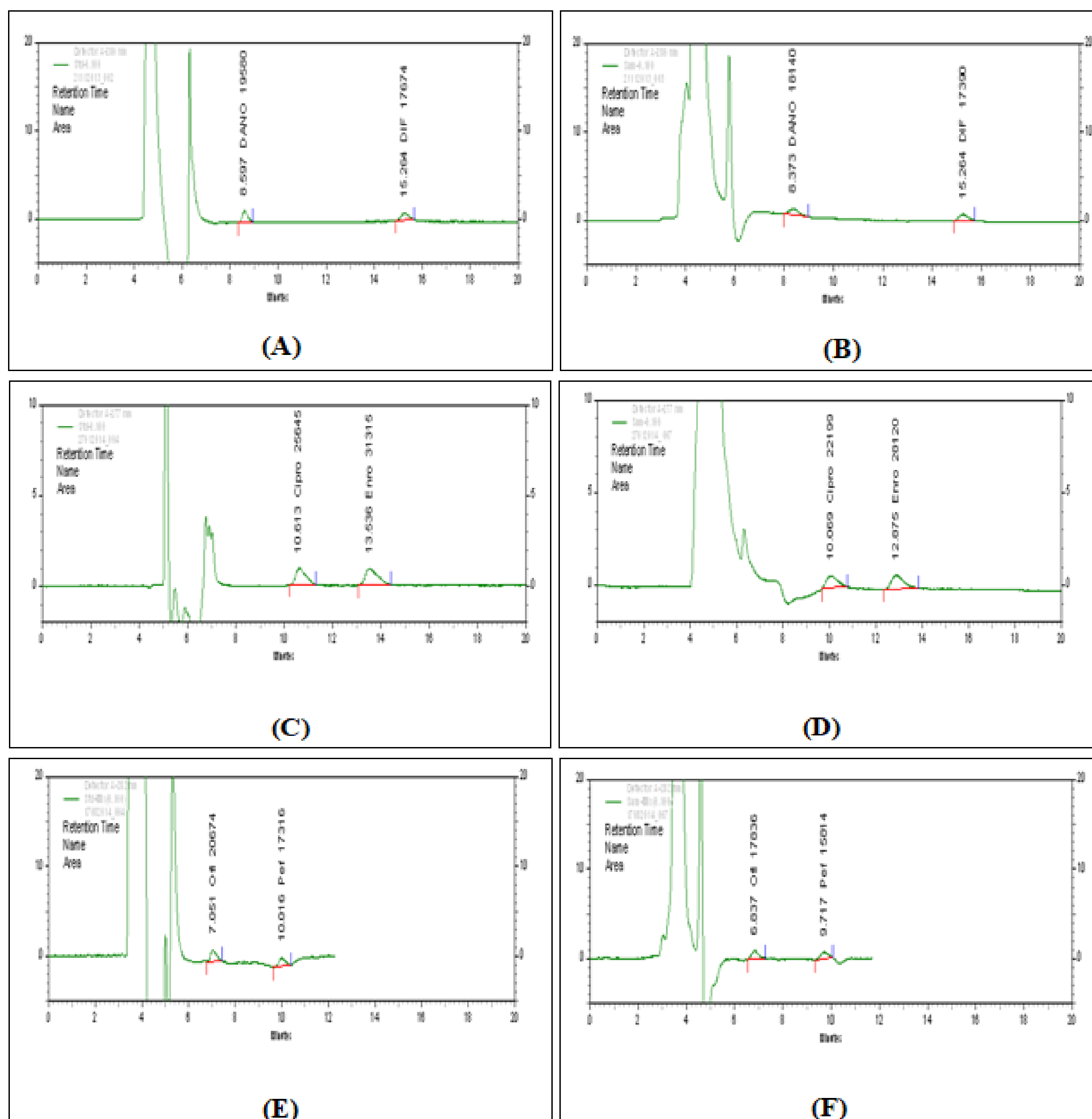

FIG. 2: CHROMATOGRAM FOR THE: (A), (C) AND (E) MIXTURE OF BOTH DRUG STANDARDS AT 100 NG/ML CONCENTRATION LEVEL AND (B), (D) AND (F) SPIKED CHICKEN TISSUES HOMOGENATE AT SAME CONCENTRATION LEVEL

CONCLUSION: All analytical methods were successfully developed to determine selected fluoroquinolones in chicken muscle by high-performance liquid chromatographic technique. Improved sample extraction protocols were developed to extract the analytes from the chicken tissues with good recoveries (above 87%). The correlation coefficient of the linearity curves for all drugs was above 0.9700. The detection and quantification limits were found to be low enough to determine fluoroquinolones residues in chicken meat. The analytical performance of the methods with different parameters was determined, and obtained results were good and within limits. These developed methods can be applied for routine determination of the fluoroquinolones in chicken tissue samples.

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