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ANALYSIS OF SOME RESIDUAL ANTIBIOTICS IN MUSCLE, KIDNEY AND LIVER SAMPLES OF BROILER CHICKEN BY VARIOUS METHODS

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Abstract: The present study was conducted for the analysis and comparison of selected residual antibiotics in broiler chicken available in local market. The broiler samples included muscle, kidney and liver. The quinolones included in this study were, oxolinic acid, nalidixic acid, flumequine, enrofloxacin, norfloxacin and ciprofloxacin. An assessment of variation of these analytes (residues) in these samples was made. The intertissue/organ comparison within each analytical technique and intermethod comparison of results obtained by HPLC, UV spectroscopy and ion association complex techniques were made. TLC was used to separate and identify the quinolone residues. Infrared (IR) was also used for identification of the residues. HPLC with ODS column and UV detector and UV/ visible spectroscopy were used for quantification of the residues. Oxolinic acid, nalidixic acid and norfloxacin residues were absent in all the samples. The "ciprofloxacin–brilliant blue G" ion association complex was used for ciprofloxacin determination using ion association complex technique. Good compatibility of the spectrophotometric results was found with those of high pressure liquid chromatography.

Keywords: Maximum Residue Limits (MRL's), Ion Association Complex Technique (IAC) and HPLC.

Introduction

Recently the scale of growth of chicken, swine and fish has expanded throughout the world. The density of the chicken, swine and fish growth is so high at farms that infection often occurs. Large amount of quinolone antibacterials are applied in poultry industry, to prevent infectious diseases. The poultry birds are treated with quinolone antibiotics, which inhibit the DNA gyrase formation [1,2]. Ciprofloxacin (CPRF), enrofloxacin (ENRF), norfloxacin (NRF), flumequine (FLUM), nalidixic acid (NAL) and oxolinic acid (OXOL) are the fluoroquinolones/quinolones frequently applied to treat the diseases. These drugs treat gonorrhoea, bacterial gastroenteritis, skin and soft tissue infections, complicated and uncomplicated urinary tract infections caused by gram positive and gram negative organisms. These drugs find extensive applications in the field of medicine and chemical analysis [3].

The quinolones have been classified according to their antibacterial spectrum; potency and pharmacology. There is no widely accepted classification at present [4,5]. These are divided into two categories. The first generation quinolones include, NAL, OXOL, FLUM and piromidic acid (PIRM), which have good antibacterial activity against gram negative bacteria [6]. Their antibacterial effect is no longer good as drug resistant bacteria have evolved [7]. The second category (the second and third generation) includes fluoroquinolones containing fluorine at C-6 position and piperazinyl

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ring at C-7 position, such as marbofloxacin (MARB), ofloxacin (OFL), danofloxacin (DNF), ENRF, sarafloxacin (SRF), NRF and levofloxacin (LEVOF). These have broad antibacterial spectrum. They are safe drugs and are effective against gram positive, gram negative bacteria and mycoplasma. So their antibacterial activity is good [3,7,8].

The ever increasing use of quinolones, fluoroquinolones in poultry industry has caused their residual deposition in the poultry products resulting in the drug resistant bacteria. It has become a matter of foremost importance to screen the poultry birds of these residual antibiotics, down to the safer MRL's (Maximum Residue Limits) set by international fora Table 1 [9,10,11]. Various analytical techniques such as atomic absorption, spectrometry, polarography, AC-oscillopolarographic titration, differential pulse polarography, capillary zone electrophoresis, spectrofluorometry and high performance liquid chromatography have been used for determination of these drugs. But these techniques are either very expensive or not available at all at most poultry products quality control laboratories. The ultraviolet spectroscopy and other spectrophotrometic techniques are the most preferred and easy ones for assay of the different drugs in biological samples. Both these methods are simple and easy to apply. The present communication is aimed at testing the validity of these techniques in the quantificatin of some residual antibiotics extracted from different organs of broiler birds.

Materials and Methods

The samples were collected randomly from local markets situated in Lahore. The broiler birds came from the broiler poultry farms situated in the outskirts of Lahore. The samples were collected during the summer of 2004.

The quinolones were extracted, purified with

Table 1.
Maximum Residue Limits (MRL's) for quinolones in force in Europe [9,10,11].

Substance	Marker Residue	Species	MRL (μg/kg)	Tissue
Enrofloxacin	Sum of	Bovine	30	Muscle
	enrofloxacin &	Porcine	30	Liver
	ciprofloxacin	Poultry	30	Kidney
Ciprofloxacin	Sum of	Bovine	30	Muscle
	enrofloxacin &	Porcine	30	Liver
	ciprofloxacin	Poultry	30	Kidney
Flumequine	Flumequine	Bovine	50	Muscle
		Ovine	100	Liver
		Porcine	300	Kidney
		Poultry	50	Fat, Skin
		Salamander	150	Muscle, Skin

bond elute cartridges and separated from one another on large TLC separating plates spread with the silica gel and cellulose using various solvent systems such as Me_2CO: Et_2O (7:3 V/V) with silica gel plates and BuOH: isopropyl alcohol: H_2O (4:4:2 V/V), H_2O: BuOH: HOAc (5:4:1 V/V), MeCN: H_2O: Me_2 CO:α-Chlorohydrin (70:2.5:10:5.25 V/V) and $CHCl_3$: MeOH: NH_4OH (2:1:1) with the cellulose plate [12]. NRF, OXOL and NAL were absent. Known fractions of total volume of each sample were used for TLC.

The UV spectroscopy and ion association complex spectrophotometric techniques were then applied which are highly accurate and selective methods. Their limits of detection were generally sufficient for determination of residues. HPLC was used as the standard method to validate these. The IAC spectrophotometric technique was restricted only to NRF and CPRF. It selectively forms blue coloured chloroform-soluble ion association complexes due to their interaction with brilliant blue G (BBG) in NaOAc-AcOH buffer of pH 4 [13].

Extraction of the Residues from Broiler Tissues

Broiler liver and muscle samples (10g each) and 5g samples of broiler kidney were used for extraction of quinolones. Phosphate buffer with acetonitrile was added to each sample with magnetic stirring. The extracts were filtered by whatman filter paper. This procedure was repeated thrice for each sample. Then activated charcoal was used for decolouration and anhydrous sodium sulphate was used for dehydration of the sample extracts. Defatting was done by n-hexane saturated with acetonitrile in a separation flask. Each sample was made up to mark to 50ml by addition of deionised water. Cleaning of the basic fluoroquinolones was performed with a bond elute (Varian, Walton-on-Thames, UK) strong cation exchange (SCX) solid phase extraction cartridge and that of acidic quinolones with AGMP-1 resin (Bio-Rad Richmond, CA, USA); anion exchange cartridge.

Preparation of 0.05% Chromogen Brilliant Blue G (BBG) Solution

Stock solution of BBG was prepared by dissolving 50mg of BBG in a few drops of acetone and made up to mark (100ml) in a measuring flask with double distilled water.

Preparation of Ion Association Complex Standards of ciprofloxacin and norfloxacin

A series of working standards of 1, 2, 3, 4, 5, 6 ppm for CPRF and 2, 4, 6, 8, 10 ppm for NRF were prepared from the 100 ppm stock standard by taking their respective volumes in ml equal to their respective ppm's and making them up-to mark in 100 ml measuring flasks. One ml from each flask was taken into a series of 125 ml separating flasks. A sodium acetate / acetic acid buffer of pH 4 was prepared and 5 ml of this buffer was added into each flask having CPRF and 2 ml of it into each separating flask having NRF was added. To each of the flasks for both CPRF and NRF, 1 ml of 0.05% chromogen BBG was added. Then 10 ml chloroform was added into each flask and shaken well. The chloroform layer was dried by running it into anhydrous sodium sulfate.

Preparation of Samples for Ion Association Complex (IAC) Technique

The NRF scratchings NRF spots of each sample on TLC plates were taken to dissolve them in a few drops of acetone. But NRF was not located in any of the 15 samples of layer liver, kidney and muscle. CPRF scratchings of each sample was dissolved in 1 ml of deionised water. 5 ml of the buffer were added followed by 1 ml BBG into each flask. Then 10 ml chloroform was added into each flask and shaken well. Chloroform layer was retained, dehydrating it with anhydrous sodium sulfate.

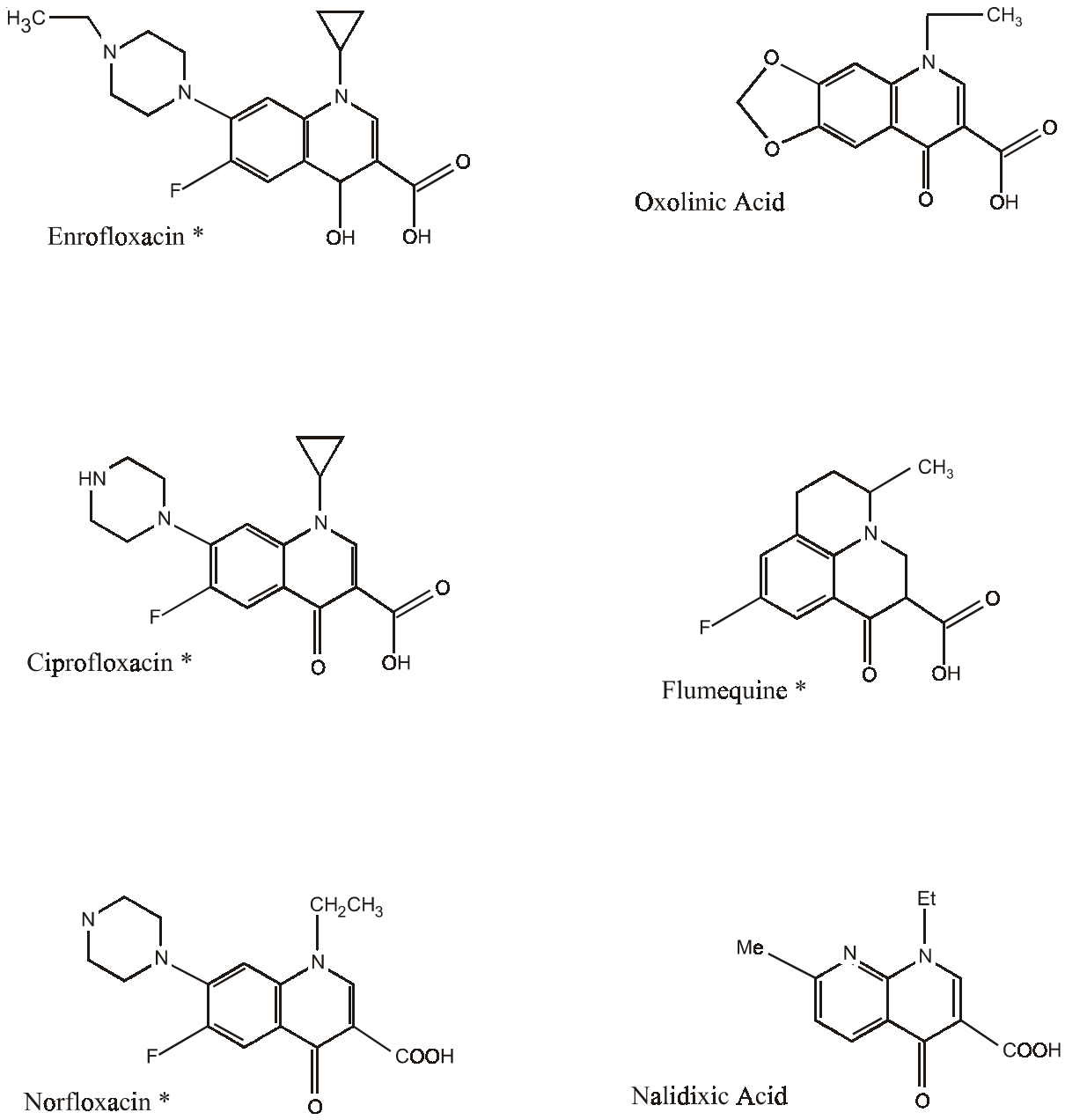

Fig 1. Chemical structures of quinolones (* = fluoroquinolones) involved in residual analysis.

Analysis of the (IAC) Samples

The absorbance of the standards and samples of CPRF were recorded at 610 nm and those for NRF standards only (as NRF was absent on TLC plate) at 614 nm. Standard calibration curves were plotted and concentrations of samples of CPRF from layer's liver, kidney and muscle were calculated. The minimum limits of detection for CPRF and NRF were 0.5 and 0.4 ppm and those maximum were 6 and 8 ppm respectively.

Analysis by HPLC

HPLC systems consisted of LC-9A pump (Shimadzu), SPO-6AB, UV/visible spectro-

photometric detector (Shimadzu), CTO 6A oven (Shimadzu), SCL-6B system controller, and CSW-32 software. The ultrasonic cleaner (EYELA Tokyo Rikakai Co. Ltd.) was used for degassing the samples, standards and mobile phase. Sartorius filtration assembly (Germany) was used for filtration with nylon filter of 0.45 ìm pores (Germany). The pH of the mobile phase was set by pH meter (Hanna HI-8418). The extractants, solvents and mobile phase constituents were of HPLC grade.

The standards of 5, 10, 15, 20, 25, 30, 35 and 40 ppm were prepared in HPLC mobile phases which were also used for UV analysis. The mobile phase consisted of MeCN: phosphate buffer (35:65 v/v) added with 3.5 mM SDS. The wavelengths used were 279, 284 and 241 nm for CPRF, ENRF and FLUM respectively. The injection volume was 20 ìl, the detector worked at ambient temperature and flow-rate 1 ml per minute for CPRF and ENRF and 1.5 ml per minute for FLUM. The mobile phase for FLUM consisted of 0.01 M Oxalic Acid: Acetonitrile: methanol (6:3:1). As oxalinic acid (OXOLN), NAL and NORF were not identified in TLC technique, these were not estimated on HPLC.

Analysis of Samples by UV Technique

The samples from HPLC analysis were taken off, dried gently on water bath, separated by TLC, spots for CPRF, ENRF and FLUM were scratched and redissolved in the respective mobile phases of HPLC, and working standards from HPLC technique were used. Absorbance was recorded on U-2000 Hitachi Spectrophotometer Tokyo (Japan) at 279, 284 and 241 nm for CPRF, ENRF and FLUM respectively. Calibration plots were drawn and sample residue concentrations were calculated.

Identification and Quantification of Residues

Quinolones (NRF, CPR, ENRF, OXOL, NAL, and FLUM) were identified in the samples by comparing their IR spectra, migration values and retention times of standards with those of unknown substances in the samples using their respective mobile phases in case of HPLC and UV detection. Estimations were made (1) by using standard calibration curves, and (2) by using the relation of "peak area /ppm" calculation from the standard calibration curve values of the peak area and concentrations. NRF, NAL and OXOL were not located and identified by TLC technique. These could not be identified by IR as well. Hence these were absent. Only FLUM, ENRF and CPRF were present.

Statistical Treatment

The statistical treatment given to the analyzed data were (i) F test, (ii) Student "t" test, and (iii) comparison of correlation coefficients of the analyzed residues. All the three statistical treatments of the analyzed residual data validate the test techniques with reference to an accepted reference technique (HPLC). The correlation coefficient is used as a measure of correlation between two sets of data (residues).

A correlation coefficient "r" can be calculated for a calibration curve to ascertain the degree of correlation between them. As a general rule, $0.90 < r < 0.95$ indicates a fair correlation, $0.95 < r < 0.99$ a good correlation, and $r > 0.99$ indicates excellent correlation. An $r > 0.99$ can sometimes be obtained with care. "r" may range from +1 to –1. It is common practice in the clinical chemistry literature when comparing two methods to analyze a series of samples over a range of concentrations by both methods and calculate a correlation of results of one method with those of the other. In this paper the correlation coefficients between the residual data obtained by UV and IAC techniques were separately calculated. The coincidence between the "r" values with respect to sign and numerical value shows the degree of agreement and conformity between the

two techniques. Hence this treatment gives us a finger print comparison between the test and reference techniques. F test and "t" test decide that the test technique differs within permissible limits internationally accepted. The correlation coefficient gives a finger print agreement of a test technique (UV and IAC) with the reference technique (HPLC). Hence the numerical value of correlation coefficient holds the same statistical significance as the F test and "t" test [14].

Results

The results (Table 2) show average residual concentration of antibiotics; CPRF, ENRF and FLUM in broiler samples. FLUM was not found in muscle but was detectable to a small extent in liver and kidney. CPRF was detected in liver, kidney and muscle but to a lesser extent as compared to ENRF. There was slight intra-analyte residual difference between the UV and IAC test methods and standard HPLC method. The concentration of the residues of various antibiotics in various samples according to HPLC and UV in decreasing order was: CPRF: BL>BK>BM, ENRF: BK>BL>BM, FLUM: BK>BL (FLUM was absent in broiler's muscle). The order of concentration of CPRF residues according to IAC technique was: BL>BK>BM. OXOLN, NAL and NRF were absent in all samples. Moreover the level of concentration of these quinolones was dependant upon their lipophylicity. Thus the more lipohyhyllic ENRF was in higher concentration and the less lipophyllic CPRF was in lower concentration. The least lipophyllic FLUM had the least concentration.

Discussion

The validity of the UV and IAC techniques was established by "F" test and "t" test of the results. Table 3 shows F-Test data for the test techniques (UV and IAC). The tabulated F-Test value for 4 degrees of freedom is 6.39 and all the calculated values in the Table are below this value, showing that variances of both methods (UV and IAC) do not have statistically significant differences from those of the standard method (HPLC) for the intra-analyte residues of all the three broiler samples. It also means that the standard deviations are due to manual handlings and not due to the determinate errors including those in sample preparation. Hence student "t" test was performed to test the validity of both the test methods. Table 4 shows the student "t" test values for both the test techniques for CPRF residues and for only one technique (UV spectroscopy) for ENRF and FLUM residues involving liver, kidney and muscle samples. All the calculated values (2.776) for 4 degrees of freedom and 95% confidence level.

Table 2.

Mean residual antibiotic concentration (mg/kg) in the liver, kidney and muscle of broiler chicken.

Antibiotic residues	HPLC Technique			UV Technique			IAC Technique		
	Broiler Liver	Broiler Kidney	Broiler Muscle	Broiler Liver	Broiler Kidney	Broiler Muscle	Broiler Liver	Broiler Kidney	Broiler Muscle
CPRF	250.25	146.08	87.81	24.49	140.32	85.29	245.34	143.602	85.39
ENRF	1430.41	2143.79	709.87	1425.86	2136.88	703.72	*N.D.	N.D.	N.D.
FLUM	30.60	52.4	Nil	29.65	50.97	Nil	N.D.	N.D.	N.D.

Hence both the test techniques were statistically valid, at the same time not differing significantly from the standard (HPLC) technique.

Tables 5 shows intra-residue inter-organ correlation coefficient for HPLC, UV and IAC techniques. For example in case of CPRF estimated from broiler liver and kidney, the values of "r" were -0.7949, -0.6972 and -0.7672 for the above three techniques respectively. All the three "r" values were negative and of about the same order. But in case of ENRF and FLUM estimated from different organs, the two techniques HPLC and UV show "r" values of the same magnitude and sign. Table 6 shows intra-organ inter-residue correlation coefficient in which the same organ has the same value and sign even if estimated under different techniques. For example, for broiler kidney the "r" values for ENRF/CPRF residues under UV and HPLC techniques were – 0.18292 and -0.17569. Hence there was very fine complementarity and symmetry of the sign and the degree of the correlation, proving the validity of the test techniques according to the internationally acceptable standards. The higher CPRF residual concentrations in liver as compared to these in kidney and muscle are attributable to (i) the metabolite formation from ENRF to CPRF by de-ethylation, (ii) higher rates of excretion of CPRF from the kidney as compared to that of ENRF due to its lower lipophylicity than ENRF (iii) the lower capacity of binding of CPRF than that of ENRF and (iv) unsophisticated amounts of dosages administered to the poultry birds. In case of inter-tissue/organ difference of ENRF residues the relatively lower

Table 3.

F-Test values for the two test techniques (UV and Ion Association Complex Techniques).

Antibiotic	**IAC Technique**			**UV Spectroscopy**		
Residues	Broiler Liver	Broiler Kidney	Broiler Muscle	Broiler Liver	Broiler Kidney	Broiler Muscle
CPRF	1.2417	1.0654	1.3820	1.1766	1.0720	1.0572
ENRF	N.D.	N.D.	N.D.	1.3356	1.0020	1.0266
FLUM	N.D.	N.D.	N.D.	1.0591	1.0552	Nil

ND: not detectable

Table 4.

The student "t" test values for the UV and IAC Techniques.

Antibiotic	**IAC Spectroscopy**			**UV Technique**		
Residues	Broiler Liver	Broiler Kidney	Broiler Muscle	Broiler Liver	Broiler Kidney	Broiler Muscle
CPRF	1.4855	0.5008	0.8417	1.5	0.2148	0.9249
ENRF	0.02701	0.01353	0.11964	N.D	N.D	N.D
FLUM	0.04763	0.03946	Nil	N.D	N.D	N.D

Table 5.
Correlation coefficients for residual antibiotics estimated in broiler liver, kidney and muscle using different analytical techniques.

Antibiotic Residues	Inter-Tissue Correlation Correlation	HPLC Technique	UV Spectroscopy	IAC Technique
CPRF	Broiler Liver/Broiler Kidney	-0.7949	-0.6972	-0.7672
	Broiler Muscle/ Broiler Liver	-0.7672	-0.754	-0.75497
	Broiler Muscle/ Broiler Kidney	+0.8588	+0.8365	+0.8427
ENRF	Broiler Liver/Broiler Kidney	+0.701935	+0.496378	N.D.
	Broiler Muscle/ Broiler Liver	+0.7741	+0.787252	N.D.
	Broiler Muscle / Broiler Kidney	-0.12089	-0.10502	N.D.
FLUM	Broiler Liver/Broiler Kidney	+1.00	+0.994871	N.D.
	Broiler Muscle/ Broiler Liver	Nil	Nil	N.D.
	Broiler Muscle / Broiler Kidney	Nil	Nil	N.D

Table 6.
Intra-organ inter-residual coefficient of correlation.

Inter-residual Correlation	UV Spectroscopy			HPLC Technique		
	Broiler Liver	Broiler Kidney	Broiler Muscle	Broiler Liver	Broiler Kidney	Broiler Muscle
ENRF/CPRF	-0.76444	-0.18292	+0.369166	-0.72765	-0.17569	+0.433429
ENRF/FLUM	+0.023683	-0.3628	Nil	+0.030345	-0.34816	Nil
CPRF/FLUM	-0.05929	-0.32938	Nil	+0.054266	-0.32809	Nil

ENRF in liver is attributable to its primary metabolite formation [15] and its higher kidney residue levels are due to its higher lipophylicity [16]. The decreasing order of residual amounts of quinolones was ENRF>CPRF>FLUM. This is exactly in accordance with their decreasing order of lipophylicity as shown by their log D values. The decreasing order is also in accordance with the residual amounts of quinolones in kidney, liver and muscle [17]. The essence of the work is that the results obtained by test techniques do not differ significantly from the reference (HPLC) technique thereby providing authentication of these easily applicable and cheaper techniques.

This work enables the common laboratories to monitor the quinolone residues in poultry products. The validation tests "F test" and "t test" have shown the test techniques (UV and IAC) to be statistically equivalent for the determination of the residues. Also,

the value, sign and symmetry of correlation obtained by different test techniques show their degree of accuracy and equivalence with the reference technique. Those samples are deviating samples which have some residue larger that MRL's set internationally. The ENRF and CPRF in the analyzed samples were higher than MRL's. However these samples do not have any residual NAL and NORF. FLUM residues were detected only in liver and kidney of the broiler birds and not in the broiler muscle samples. The FLUM residual level of kidney and liver was below safer maximum residue limits internationally accepted (Table 1). These should be strictly monitored by sufficient washing time periods. The samples should also be monitored for the quinolone resistant pathogenic bacterial species related to the respective residue.

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