OPTIMIZATION AND VALIDATION OF QUINOLONES DETERMINATION IN POULTRY MEAT BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND SECONDARY CONFIRMATION WITH FOURIER TRANSFORM INFRARED SPECTROPHOTOMETERY

By

Shumaila Asif

Department of Biochemistry
Faculty of Biological Sciences
Quaid-e-Azam University
Islamabad

OPTIMIZATION AND VALIDATION OF QUINOLONES DETERMINATION IN POULTRY MEAT BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND SECONDARY CONFIRMATION WITH FOURIER TRANSFORM INFRARED SPECTROPHOTOMETERY

Shumaila Asif

M.Phil. Biochemistry

Department of Biochemistry
Faculty of Biological Sciences
Quaid-e-Azam University
Islamabad

CERTIFICATE

This thesis, submitted by **Ms. Shumaila Zulfiqar** to the Department of Biochemistry, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan, is accepted in its present form as satisfying the thesis requirement for the Degree of Master of Philosophy in Biochemistry/Molecular Biology.

Supervisor: Dr. Salman Akbar Malik

External Examiner: Dr. Asif Mir

Chairman: Dr. Salman Akbar Malik

Dated: February 10, 2011 .

Table of Contents

ACKNOWLEDGEMENTS

Ups and Above every thing all glory to the " ALMIGHTY ALLAH".
The Most Gracious, The Most Merciful.

I offer my thanks from the deepest core of my heart to **Holy Prophet Muhammad (PBUH)** who is forever a touch of guidance and knowledge for humanity as a whole. In the first place I would like to record my gratitude to Prof. **Dr. Salman A. Malik**, Head of Biochemistry Department, for his supervision, advice, and guidance from the very early stage of this research as well as giving me extraordinary experiences throughout the work. Above all, he provided me unflinching encouragement and support in all aspects. His truly scientist intuition has made him as a constant oasis of ideas and passions in science, which exceptionally inspired and enriched my growth as a student, a researcher. I express my heartiest and sincerest sense of gratitude to all the members of my Supervisory Committee for their contribution in my studies.

I express my heartiest and sincerest sense of gratitude to **Dr. Qurban Ali**, Direct General, National Veterinary Laboratories (NVL) and **Dr. Muhammad Javad Arshad**, Senior Scientific Officer, NVL, for their guidance, valuable suggestions and encouraging attitude during the course of this study. I am also very thankful to **Dr. Muhammad Asif**, Scientific Officer, NVL, for his technical assistance and cooperation during my research work which no doubt helped me in attaining my destiny.

My parents deserve special mention for their inseparable support and never ending prayers; my late father, **Zulfiqar Hussain** (may Allah rest his soul in peace) in the first place is the person for showing me the joy of intellectual pursuit ever since I was a child, my mother, **Tabassam Anwar**, who sincerely raised me with her caring and loving

nature, and last but not the least special thanks to my little daughter Eshal whose innocence and mischief keep me alive and smiling.

Shumaila Asif

List of Figures

Summary

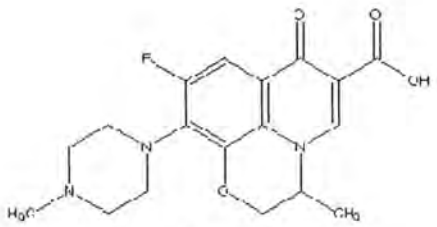
A multiresidue method for the determination of a range of eight different quinolones including ofloxacin (OFL), norfloxacin (NOR), ciprofloxacin (CIP), enrofloxacin (ENR), sarafloxacin (SAR), difloxacin (DIF), oxolinic acid (OXO), and flumequine (FLU) was optimized and validated. The method used 0.1% trifuoroacetic acid in methanol followed by solid phase extraction with C_{18} cartridges for the maximum recovery and purification of analytes. Chromatographic separation and detection was achieved using C_8 HPLC column with a mobile phase consisting of 10 mM citric acid under gradient mode. A good limit of detection (LOD) and quantification (LOQ) were achieved which varied between 3.74 to 9.10 and 12.47 to 30.34 respectively and were below the maximum residue limits (MRLs). The method was linear for all the quinolones under a wide range with correlation coefficients between 0.9989 and 0.9997. Once the method was setup, this was followed by a survey for the detection of quinolones residues in poultry meat samples which were collected from various local poultry shops in Rawalpindi and Islamabad. Overall, out of 60 samples tested, 11.7, 36.7, 48.4, 48.5, 10, 8.4, 6.7 and 15% samples were found to be contaminated with OFL, NOR, CIP, ENR, SAR, DIF, OXO and FLU respectively with 29.1, 77.3, 84.9, 50, 0, 33.3 and 32.5% of these samples being above MRL set by EU/FAO. For secondary confirmation all the samples tested with HPLC were also analyzed by fourier transform infrared spectrophotometer (FTIR). A good correlation was found between the HPLC and FTIR results, however, 7 of 60 (11.7%) samples detected with HPLC were found negative with FTIR. The presence of a large proportion of quinolones residues in poultry meat is a concern and requires attention by the legislative bodies and calls for raising of awareness among the farmers and other people linked to this industry. This will not only protect consumer health but also will have positive impact on export of poultry meat.

In recent years there has been extensive growth and proliferation of poultry industry in Pakistan. This expansion has, o f course, encountered proportional increase in supportive medication particularly antibiotics which are not only used as therapeutic agents abut also for prophylaxis and as growth promotors (Javed, 1988). Among these, quinolones are widely used. These antibacterial agents are not cleared from the body rapidly and therefore become a part of tissues in the form of residues. Residues of veterinary medicinal products are "pharmacologically active substances (whether active principles, excipients or degradation products) and their metabolites which remain in foodstuffs obtained from animals to which the veterinary medicinal product in question has been administered" (www.noah.co.uk). Consumption of these residues poses threats for human beings in various ways.

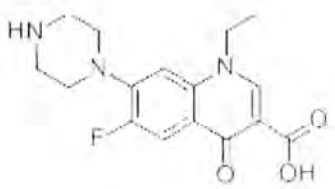
Unfortunately many of quinolones especially fluoroquinolones are commonly used in human and veterinary medicine which is a serious matter and is further intensified by the fact that these are used irrationally and unprescribed. A large number and variety of quinolones are available in Pakistan both in human and veterinary medicine. These are even used prophylactically. Although there are no exact figures for Pakistan but in neighbouring country China the annual consumption of quinolones has been estimated to be 470 tonnes in animals compared to 1350 tonnes in humans. The figures may be more or less similar in Pakistan where the medicines are used irrationally and unlimited without check and balance as there is no legal framework in execution for prescription of veterinary medicines (WHO/EMC/ZDI/98.10, 1998).

Quinolones

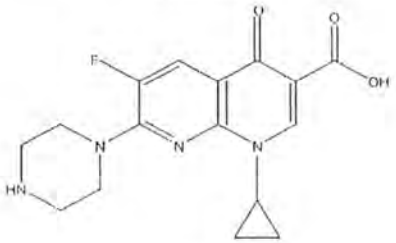
Quinolones are a group of potent synthetic antibiotics that work by interfering with uncoiling mechanism of bacterial DNA. The first member of this group was nalidixic acid which was presented for use in 1962. It was used initially to treat urinary tract infections. A major subgroup of quinolones is fluoroquinolones that contain a fluorine at central ring usually at sixth or seventh position (Nelson *et al.*, 2007; Ivanov and Budanov, 2006; Hooper, 2001) (Fig-1).



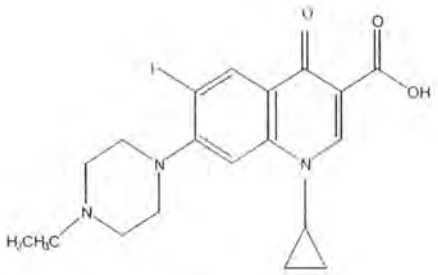
Ofloxacin



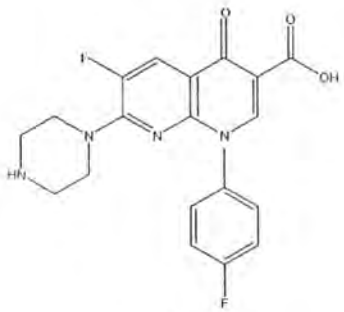
Norfloxacin



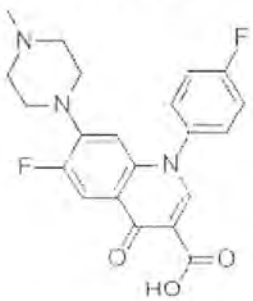
Ciprofloxacin



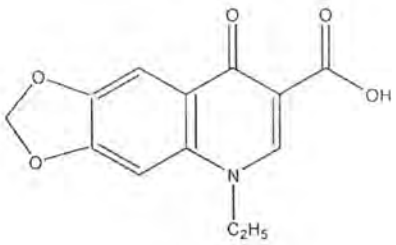
Enrofloxacin



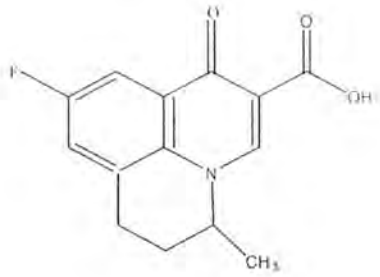
Sarafloxacin



Difloxacin



Oxolonic acid



Flumequine

Fig-1: Structures of various quinolones

Nalidixic acid was first of this group to be introduced which actually gave birth to all the predecessors and now there are second, third and even fourth generations. First generation also included oxolinic acid, cinoxacin and pipemidic acid. These were, however, not significantly improved than nalidixic acid (Norris and Mandell, 1988).

Quinolones gained wide attention by the researchers in an attempt to create some highly efficient derivative and its because of this reason that more than 10,000 analogs have been created but unfortunately very few have actually been approved for use (Childs, 2000). In general quinolones are more toxic antibiotics under use today than other antibiotics (www.mombu.com).

Indications

Fluoroquinolones are usually used for treating infections of urinary tract and those of genital origin. Since these are in general quite potent so these are used when other simple antibiotics fail or when a more aggressive response is required in serious conditions (Liu and Mulholland, 2005). Table-1 details some uses and indications of fluoroquinolones (www.merckmanuals.com).

Fluoroquinolones are active against a number of pathogens including *Neisseria* sp, *Haemophilus influenzae, Moraxella catarrhalis, Mycoplasma* sp, *Chlamydia* sp, *Chlamydophila* sp, *Legionella* sp, Enterobacteriaceae, *Pseudomonas aeruginosa* (particularly ciprofloxacin), *Mycobacterium tuberculosis,* Some atypical mycobacteria, Methicillin-sensitive staphylococci. Fluoroquinolones are contraindicated in children as they can cause damage to growth plates so affecting normal growth of bones (www.merckmanuals.com).

Harmful effects

Quinolones have usually no serous side effect but occasionally may be associated with dangerous consequences (De Sarro and De Sarro, 2001; Owens and Ambrose, 2005). These include upper gastrointestinal irritation, Effects on central nervous system (headache, drowsiness, insomnia, dizziness, mood alteration), seizures (rare, but still care required in patients with CNS disorders, tendinopathy, including rupture of the Achilles tendon, prolongation of QT-interval with eventual cardiac arrest. Also their use is linked to *Clostridium difficile*–associated diarrhea (pseudomembranous colitis) (www.merckmanuals.com).

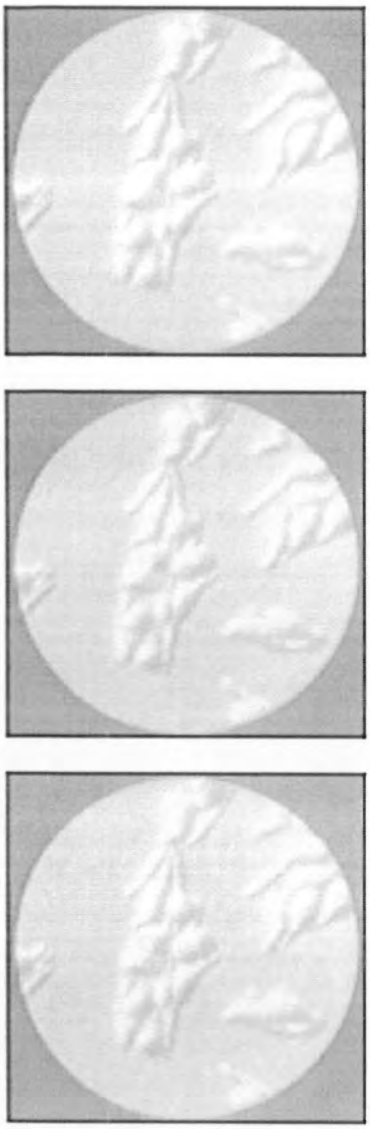
Table-1: Important uses of quinolones

Drug	Use	Comments
Fluoroquinolones except moxifloxacin	UTIs when *Escherichia coli*resistance totrimethoprim/ sulfamethoxazole is > 15%	Drugs of choice; however, increasing resistance of *E. coli* in some communities
Fluoroquinolones	Bacterial prostatitis	---
	Salmonella bacteremia	---
	Typhoid fever	Usually effective
	Infectious diarrhea	Effective against most bacterial causes (*Campylobacter* sp, salmonellae, shigellae, vibrios, *Yersinia enterocolitica*); however, increasing resistance of *C. jejuni* in some regions Not used for *E. coli* 0157:H7 Not effective against*Clostridium difficile*
Ofloxacin	Chancroid	3-day course
	Chlamydia trachomatis infections	7-day course
Newer fluoroquinolones	Community-acquired pneumonia	Other drugs preferred if patients have taken fluoroquinolones recently
	Legionella pneumonia	Drugs of choice (orazithromycin)
Ciprofloxacin	Hospital-acquired pneumonia	Used empirically because it is effective against *Pseudomonas aeruginosa* Usually used with another antipseudomonal drug
	Long-term oral treatment of gram-negative bacillary or*Staphylococcus aureus*osteomyelitis	-
	Meningococcal prophylaxis	-
	Anthrax prophylaxis	Used extensively during 2001 after bioterrorist attack in US

Mechanism of action

Fluoroquinolones work by inhibiting Topoisomerase II and IV these are actually enzymes of DNA metabolism in bacteria; the former one is also called DNA gyrase. These enzymes represent either the primary or secondary target of antimicrobial action (Fig-2). In Gram-negative bacteria, such as *E.coli*, fluoroquinolones predominantly inhibit DNA gyrase, whereas for Gram-positive organisms like Staph. aureus, Topoisomerase IV was recently found to be the principle target. DNA gyrase and Topoisomerase IV have a very similar protein structure, each composed of two subunits (Gyr-A and Gyr-B). Their principal function is different: The so-called DNA

gyrase introduces negative supercoils into the linear DNA double helix, which results in the highly condensed 3-dimensional structure of the genetic material usually present inside the cell. This mechanism is necessary to condense the bacterial chromosome. In *E. coli*, for example, a DNA strand of around 1.300 µm length must fit into a cell which is only 2 µm long. The function of Topoisomerase IV is barely understood. However, it is known that this enzyme is involved in the separation process of the DNA daughter chains after chromosome duplication. Models to explain the activity of quinolones at the target site at present only exist for DNA gyrase. The Gyr-A subunits of this enzyme were proposed to initially bind to the double stranded DNA helix. In an ATP-dependent process, described as "intermediate gate opening step", both DNA strands are cleaved at certain 4 base pair staggered sites. The 5'ends of the DNA chain are thereby bound covalently to Tyrosin122 residues within the Gyr-A subunits. Gyr-B subunits are probably responsible for the ATP-dependent resealing process of the DNA. At the location described above, DNA is present as single strands, forming a bubble-shaped binding pocket. Two quinolone molecules self-assemble to form a dimer structure inside the gyrase-induced DNA enzyme pocket. They bind to the complex by electrostatic forces, which stabilizes the intermediate stage in this reaction step (Fig-3). Evidence exists that the C7- amine substituents of quinolones additionally interact with proposed "quinolone binding pockets", located at the Gyr-B subunits, in order to further strengthen the attachment to the drug-DNA-enzyme complex. In this way the progress of the supercoiling procedure, which would include rearrangement of the DNA segments, reattachment and resealing of the cuts, is locked up. Permanent gaps in the DNA strands induce synthesis of repair enzymes called exonucleases, initiating uncoordinated repair processes. This results in breakdown of the DNA, leading to irreversible damage and, finally, to death of the bacterium (Leverkusen, 1999).

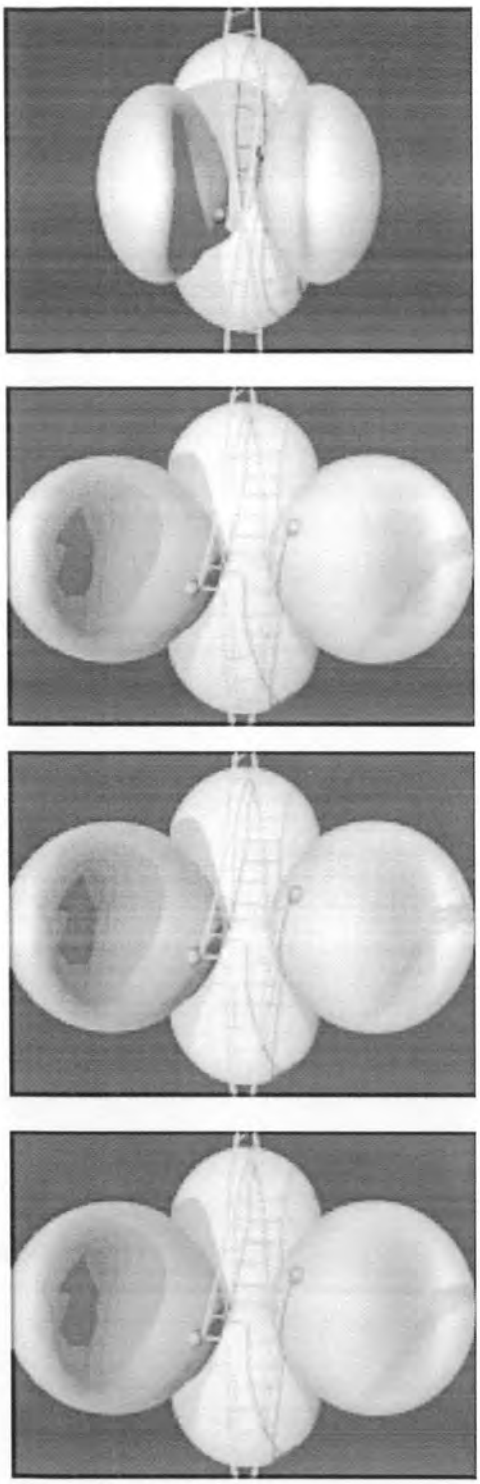


a. Intact mainly round shaped bacteria (*E. coli.*).

b. After application of enrofloxacin all the have started to swell (circular shape). Some bacteria have already bursted (spots)

c. After 7h all bacteria have bursted (spots)

Fig-2: Mechanism of action: effect on bacteria (Leverkusen 1990)



a. DNA double helix and gyrase with two A and two B subunits

b. DNA strings bind covalently to $Tyrosine_{122}$ molecules at DNA gyrase A subunits

c.DNA strings are disconnected

d. Enrofloxacin molecules bind to a "quinolone binding pocket"

Fig-3: Mechanism of action: DNA gyrase Inhibition (Leverkusen 1990)

Grouping of quinolones

Quinolones have been divided into different generations on the basis of their antibacterial spectrum (Oliphant and Green, 2002). The earlier-generation agents are, in general, more narrow-spectrum than the later ones, but there is no standard employed to determine which drug belongs to which generation. More frequently these have been divided into various generations (Oliphant and Green, 2002; Ambrose and Owens, 2000).

First-generation

The first generation is rarely used today. It includes cinoxacin, flumequine (genotoxic carcinogen), nalidixic acid (genotoxic carcinogen), oxolinic acid, piromidic acid, pipemidic acid), rosoxacin (restricted use).

Second-generation

The second-generation quinolones include ciprofloxacin, enoxacin (removed from clinical use), fleroxacin (removed from clinical use), lomefloxacin (discontinued), nadifloxacin, norfloxacin (restricted use), ofloxacin (only as ophthalmic), pefloxacin, rufloxacin.

Third-generation

Unlike the first- and second-generations, the third-generation is active against streptococci. It includes balofloxacin, grepafloxacin (removed from clinical use), levofloxacin, pazufloxacin, sparfloxacin, temafloxacin (removed from clinical use), tosufloxacin.

Fourth-generation

Fourth generation fluoroquinolones act at DNA gyrase and topoisomerase IV. This dual action slows development of resistance. These include clinafloxacin, gatifloxacin

(removed from clinical use), gemifloxacin, moxifloxacin (restricted use), sitafloxacin, trovafloxacin (removed from clinical use), prulifloxacin (Oliphant and Green, 2002).

Use of quinolones in humans

Quinolones have been widely used in human medicine. A number of quinolones have been approved and used as they are broad spectrum and therefore used for various infections specially at the time when increased resistance has been observed against many other commonly used antibiotics, quinolones emerged as no less than a blessing and valuable alternative. However, with the continuous and extensive use of quinolones since 1980s resistance has also been observed in human pathogens against these antibiotics. A single mutation on chromosome is enough to cause gross resistance which then spreads wide (WHO/EMC/ZDI/98.10, 1998).

Use of quinolones in animals

Like in human beings the first quinolone used in animals was nalidixic acid in 1960s. This was later joined by then newly introduced and highly potent fluoroqinolones. Together with the old quinolones, other and new quinolones and fluoroquinlones are being used even today including flumequine, oxolininc acid, norfloxaci, enrofloxacin etc. (WHO/EMC/ZDI/98.10, 1998). Table-2 compares regulations regarding the use of various quinolone antibiotics in use in various countries of the world. Other quinolones are also used in food animals in Pakistan which are not recommended for use in food animals in Europe and USA.

Antibiotic misuse and resistance

Resistance to quinolones can evolve rapidly, even during a course of treatment. Numerous pathogens, including *Staphylococcus aureus*, enterococci, and *Streptococcus pyogenes* now exhibit resistance worldwide (Jacobs, 2005). Widespread veterinary usage of quinolones, in particular in Europe, has been implicated (Nelson *et al.*, 2007). Fluoroquinolones have been recommended to be reserved for the use in patients that are seriously ill and may soon require immediate hospitalization.

Table-2: Quinolones licensed for use in food animals by regions of the world

Region	Livestock	Poultry	Pet animals	Fish
Europe	Enrofloxacin flumequine Marbofloxacin Danofloxacin	Enrofloxacin Difloxacin Flumequine Oxolinic acid	Enrofloxacin Difloxacin Marbofloxacin	Sarafloxacin (Oxolinic acid)[a]
USA	None	Enrofloxacin Sarafloxacin	Enrofloxacin Difloxacin Orbifloxacin	None
Japan	Enrofloxacin Danofloxacin Orbifloxacin Oxolinic acid	Enrofloxacin Danofloxacin Oxolinic acid Ofloxacin Vebufloxacin	Enrofloxacin Orbifloxacin	Oxolinic acid
Asia	Enrofloxacin Danofloxacin Ciprofloxacin	Enrofloxacin Ciprofloxacin Norfloxacin Difloxacin Flumequine Oxolinic acid Marbofloxacin Danofloxacin Orbifloxacin Ofloxacin (Sarafloxacin)	Enrofloxacin	Enrofloxacin Flumequine Oxolinic acid
Latin America	Enrofloxacin Ciprofloxacin Norfloxacin Danofloxacin (Flumequine)	Enrofloxacin Ciprofloxacin Norfloxacin Danofloxacin (Flumequine Oxolinic acid)	Enrofloxacin	Oxolinic acid
Canada	None	Enrofloxacin[b]	Enrofloxacin	None
Australia	None	None	Enrofloxacin	None
South Africa	Enrofloxacin Danofloxacin	Enrofloxacin Danofloxacin Norfloxacin	Enrofloxacin	None

[a]Substances in parenthesis are in limited use

[b]voluntarily withdrawn from market in 1998

Though considered to be a very important and necessary drugs required to treat severe and life-threatening bacterial infections, the associated antibiotic misuse remains unchecked, which has contributed to the problem of bacterial resistance. The overuse of antibiotics such as happens with children suffering from otitis media has given rise to a breed of super-bacteria that are resistant to antibiotics entirely (Linder *et al.*, 2005).

For example, the use of the fluoroquinolones had increased three-fold in an emergency room environment in the United States between 1995 and 2002, while the use of safer alternatives, such as macrolides, declined significantly (MacDougall *et*

al., 2005; Linder *et al*, 2005). Fluoroquinolones had become the most commonly prescribed class of antibiotics to adults in 2002. Nearly half (42%) of these prescriptions were for conditions not approved by the FDA, such as acute bronchitis, otitis media, and acute upper respiratory tract infection, according to a study that was supported in part by the Agency for Healthcare Research and Quality. In addition, they are commonly prescribed for medical conditions, such as acute respiratory illness, that are usually caused by viral infections (Neuhauser *et al.*, 2003).

Within a recent study concerning the proper use of this class in the emergency room, it was revealed that 99% of these prescriptions were in error. Out of the one hundred total patients studied, eighty-one received a fluoroquinolone for an inappropriate indication. Out of these cases, forty-three (53%) were judged to be inappropriate because another agent was considered first line, twenty-seven (33%) because there was no evidence of a bacterial infection to begin with (based on the documented evaluation), and eleven (14%) because of the need for such therapy was questionable. Out of the nineteen patients who received a fluoroquinolone for an appropriate indication, only one patient out of one hundred received both the correct dose and duration of therapy (Lautenbach *et al.*, 2003).

There are three known mechanisms of resistance. Some types of efflux pumps can act to decrease intracellular quinolone concentration. In Gram-negative bacteria, plasmid-mediated resistance genes produce proteins that can bind to DNA gyrase, protecting it from the action of quinolones. Finally, mutations at key sites in DNA gyrase or topoisomerase IV can decrease their binding affinity to quinolones, decreasing the drugs' effectiveness (Robicsek *et al.*, 2006; Morita *et al.*, 1998).

FDA warning:

In 2008 the US Food and Drug Administration (FDA) warned that the manufacturers of systemic fluoroquinolones must add a black box warning regarding the increased risk for tendonitis and tendon rupture. Fluoroquinolone products affected by the labeling changes include ciprofloxacin, extended-release ciprofloxacin, gemifloxacin, levofloxacin , moxifloxacin, norfloxacin and ofloxacin. All the currently marketed fluoroquinolones contain warnings regarding the risk of tendon-related adverse events

in the product labeling, including the risk of tendon rupture. This was necessary as considerable numbers of tendon-related adverse events continue to be reported despite the numerous safety labeling revisions that have been implemented since 1992. Data from the published literature suggest that Achilles tendon ruptures occur 3 to 4 times more frequently in fluoroquinolone-treated patients compared with the general population. Patients older than 60 years, those taking steroids, and kidney, heart, or lung transplant recipients are at further increased risk for these events (Ambrose *et al.*, 2000).

Maximum Residue Limit (MRL)

The maximum residue limit (MRL) is the maximum level of residues after giving a drug which is allowed in food. Medicines intended to be given to food producing animals must be used carefully keeping in view the withdrawal time which any medicine take to clear from the body and of course until this period has elapsed the animal should not be slaughtered or else the residues remain in the edible tissues and are consumed with food which, if more than the recommended MRL, can be harmful for health and also poses threats in terms of inducing resistance to pathogens which can be a serious issue and a challenge for both physicians and researchers in the development of new antibiotics (Niwa *et al.*, 2003; Horii *et al.*, 2006). Therefore, to help protect the health of consumers, MRLs have been established by different countries to ensure only residues free meat products and by products reach the consumers. For most of the drugs given to animals, MRLs have been established and by law the levels must be below or equal to this limit. MRLs are different from species to species and even from one tissue to another (www.noah.co.uk). MRLs for certain quinolones have been established by different countries and organizations like the Food and Agriculture Organization of the United Nations called Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the European Union (EU) (www.fda.gov).

Objectives of the study

The current study was conducted under various objectives which can be summarized as:

1. Optimization of method for efficient extraction of quinolones from poultry meat matrix.
2. Validation of this method for efficient extraction of quinolones from poultry meat matrix.
3. Optimization of a chromatographic method for efficient separation and identification of quinolones from poultry meat matrix by high performance liquid chromatography (HPLC).
4. Validation of this chromatographic method for efficient separation and identification of quinolones from poultry meat matrix by high performance liquid chromatography (HPLC).
5. Secondary confirmation of results with fourier transform infrared spectrophotometer (FTIR).
6. Application of optimized and validated method to the real samples.
7. Survey for determination of quinolones contamination in poultry meat from Rawalpindi and Islamabad.

The whole study was divided into two phases; in the first part procedures for detection of quinolones residues were optimized and validated while in the second half these procedures were applied to the real samples and a survey was conducted to find the presence of quinolones residues in poultry meat from local markets of Rawalpindi and Islamabad.

Chemicals and reagents:

Equipment:

- Spectrophotometer U2010, Hitachi, Japan
- High performance liquid chromatography (HPLC) system was Hitachi D-7000 Series with the following components:
    - Column Oven (L-7300)
    - Detector (L-7400)
    - Autosampler (L-7200)
    - Pump (L-7100)
    - Degasser (L-7610)
    - Interface module (D-7000) and
    - HSM Software
- Fourier transform infrared spectrophotometer (FTIR) (Thermo Nicholet, USA)
- Analytical electrical balance (Sartorius)
- Vortex shaker (IKA yellow line)
- Vacuum pump (Sartorius)
- Centrifuge machine (Sigma)
- Magnetic stirrer (Velp Scientifica, ARE)
- Electrical pH meter (Jenway)
- Solid-phase extraction apparatus SPE (Lichrolut)
- SPE cartridges C_{18}, 6 ml, 500 mg (B&J)
- Trapkit for SPE apparatus (Supelco)
- Mini vacuum pump (KNF Neuberger)
- Rotary Evaporator (BUCHI rotavapor R-200)

- Ultrasonic bath (Elma)
- Tissue homogenizer (IKA WEREKE Ultra Turrax T25 basic)

Chemicals & Reagents:

- Citric acid anhydrous ($C_6H_8O_7$) extra pure 99.5% (Scharlau AC0718)
- Disodium hydrogen phosphate anhydrous (Na_2HPO_4) HPLC grade 99.5% (Scharlau SO0345)
- Methanol (CH_3OH) HPLC grade (Fisher)
- Acetonitrile (CH_3CN) HPLC grade (Fishher)
- n-hexane HPLC grade (Scharlu)
- Trifluoroacetic acid (Fisher)
- Deionized water (purified in laboratory from distilled water).

Miscellaneous:

- Membrane filters, Polyamide, 0.45 µm, 13 mm (Sartorius)
- Glass sample vials for HPLC
- Syringes 5, 10 ml (BD)

Phase I

Setting-up of Procedure

Preliminary conditions:

Full range UV/Vis scans of standards

All the antibiotics i.e. ofloxacin (OFL), norfloxacin (NOR), ciprofloxacin (CIP), enrofloxacin (ENR), sarafloxacin (SAR), difloxacin (DIF), oxolinic acid (OXO), and flumequine (FLU) were screened by the UV/Vis spectrophotometer to work out their absorbance pattern to help set up the chromatographic conditions subsequently. The screening was, therefore, performed in a wide range (200-500 nm) and the maximum

absorbance (A_{max}) was recorded for each of the antibiotic. All the antibiotics were screened separately in graphite UV/Vis cuvettes in dissolved in mobile phase against the mobile phase as blank to get the absorbance of only the analyte.

Optimization of chromatographic conditions

Mobile phase with methanol, acetonitrile and buffer (citric acid) was used for the best elution and separation of variety of quinolones studied. Different combinations of these ingredients were tried to find the best proportion to be used under the conditions used in laboratory. The strategy used for the optimization of mobile phase was to change certain volumes at a time while keeping the others constant (Table-3 and -4).

Table-3: Strategy for mobile phase optimization

Steps	Mobile phase composition			pH
	Acetonitrile	Methanol	Buffer	
1	Variable	Constant	Constant	Constant
2	Constant	Variable	Constant	Constant
3	Constant	Constant	Variable	Constant
4	Constant	Constant	Constant	Variable
5	Constant	Constant	Constant	Constant

Table-4: Various combinations of mobile phase tried

Acetonitrile	Methanol	Buffer	Final
6	6	88	100
	12	82	100
8	6	86	100
	12	80	100
10	6	84	100
	12	78	100
12	6	82	100
	12	76	100

Also two variable pH levels of buffer were tested; 3.0 (low) and 4.5 (high). Flow rate was kept constant at 1.2 ml min^{-1}. Initially, an isocratic mode was used for the individual quinolones. Modifications were accordingly made later to get well-separated peaks. The quinolones studied were always in the following order:

$$EFL \rightarrow NOR \rightarrow CIP \rightarrow ENR \rightarrow SAR \rightarrow DIF \rightarrow OXO \rightarrow FLU$$

Initial results showed that the some peaks were not separated at pH 3.0 but at 4.5, while other would not resolve well at pH 4.5 but at 3.0, therefore, it was not possible to elute all the quinolones at the same time under isocratic conditions. For this reason a gradient program had to be used with the following schedule:

- from 0 to 12 min: ACN–methanol–buffer (pH 3.0) solution (10:12:78 v/v/v);
- from 12 to 27 min, the buffer was replaced with that of pH 4.5 and the percentage of acetonitrile was linearly increased to 40%, while methanol was maintained constant.

Optimization of procedure for analyte recovery from meat matrix:

1. Preparation of spiked samples:

The meat samples (5 g each) were spiked with different concentrations of quinolones (OFL, NOR, CIP, ENR, SAR, DIF, OXO, and FLU) to attain final concentration level of each quinolone at 50, 100 and 150 ng g^{-1} (Table-5). For the blank samples only equivalent volume of water was added. After spiking, all the samples were kept at room temperature for 30 min before freezing them overnight in refrigerator.

Table-5: Spiking of meat samples to various levels of quinolones

Meat sample used (g)	Standard solu. conc. (ng ml^{-1})	Standard solu. vol. used (ul)	Meat spike level (ng g^{-1})
5	1000	250	50
5	1000	500	100
5	1000	750	150

2. Samples preparation:

Sample preparation for residues detection involves mainly extraction of analyte from the matrix with subsequent cleanup to reduce the burden of unwanted substances and getting more refined target material. Two different extraction strategies were tried employing two different extraction buffers (phosphate and trifluoroacetic acid) initially to get adequate recovery of quinolones from meat. The remaining steps that involve mainly cleaning of sample to get rid of the unwanted matrix were kept the same.

Method I:

Sample Extraction

i. Muscle sample was chopped and placed in a 50 ml polypropylene centrifuge tube. A 20 ml volume of phosphate buffer (pH 7.4) was added to the tube and the tissue was sheared with tissue homogenizer for 30 sec at a speed of 11000 min^{-1} and left for 15 min.

ii. The homogenized tissue was sonicated for 1 min in ultrasonic processor at 0.5 cps at amplitude of 50 Hz.

iii. This was then centrifuged for 10 min at 5000 ×g at 4°C and the supernatant was transferred to a new 50 ml tube.

iv. The sediment was dissolved in further 20 ml volume of the buffer with subsequent rest for 15 min. This mixture was again centrifuged at 5000

×g at 4°C for 10 min. Supernatant was carefully removed and collected in the tube.

v. Step 4 was repeated for one last time with 10 ml buffer.

De-fatting

i. The total supernatant collected in extraction step was mixed vigorously with equal volume of n-hexane and poured into a 250 ml separatory funnel.

ii. This was left to stand until the mixture was split into clear upper organic layer containing dissolved fats and the lower aqueous layer.

iii. The aqueous layer was carefully collected and subjected to cleanup in the next step.

Sample Cleanup

This was performed by using solid phase extraction system. For this purpose, C_{18} cartridges were used. These were fitted on a glass vacuum manifold connected to an air suction pump to create vacuum inside the chamber that exerts negative pressure on the columns and suck any liquid through the column that is added on the top. A 50 ml conical flask was placed under each column as a receiving vessel to collect the flowthrough from the cartridges.

Column conditioning

The column was conditioned with 6 ml methanol followed by similar volume of water.

Sample Loading

Without drying the column, the sample extract was poured onto the column cartridge and was allowed to pass through it slowly. In order to ensure a

constant supply of test extract to the column, a PTFE (Teflon) tube was connected between centrifuge tube containing test extract and the column. The elute was discarded.

Washing of the Column

The column was washed with 3×2 ml of deionized water. When water rinse was complete, the column was allowed to dry for 2 min. The 50 ml conical flask placed under column for receiving waste was replaced by 28 ml glass universal bottle.

Elution

The quinolones were eluted from the cartridge with 2×2 ml of 1% trifluoroacetic acid in acetonitrile followed by 1 ml of acetonitrile.

Drying and reconstitution

The eluent was concentrated on rotary evaporator at 50°C. The residue was reconstituted in the mobile phase of HPLC and was filtered through 0.45 μm filter prior to analysis on HPLC.

Method II:

Sample Extraction

i. Muscle tissue was snipped and placed in a 50 ml polypropylene centrifuge tube.
ii. For the extraction of quinolones, 10 ml trifluoroacetic acid (0.1%) in methanol was added to the tube and the tissue was sheared for 30 sec with homogenizer at 11000 min^{-1}.

iii. This was allowed to settle for 15 min.

iv. The homogenized tissue was then sonicated for 1 min in ultrasonic processor at 0.5 cps at amplitude of 50 Hz.

v. This was then followed by centrifuged at 5000 ×g for 10 min at 4°C.

vi. The supernatant was transferred to a new tube.

vii. The sediment was dissolved in further 20 ml volume of the buffer solution and allowed to settle for 15 min after sonication for 1 min.

viii. This mixture was again centrifuged under similar conditions and the supernatant was carefully removed and collected in the tube.

ix. The steps 7-8 were repeated once again but with 10 ml of buffer solution.

De-fatting

i. The combined supernatant (50 ml) collected in extraction step was mixed with an equal volume of n-hexane vigorously and transferred to a separatory funnel (250 ml).

ii. This was allowed to stand so that the mixture is split into a clear upper organic layer (containing dissolved fats) and the lower aqueous layer.

iii. The aqueous layer was carefully collected.

Sample Cleanup

Sample cleanup was performed by using C_{18} solid phase extraction cartridges (6 ml, 500 mg).

Column conditioning

The column was conditioned with 6 ml methanol followed by same volume of water.

Sample Loading

Without drying the column, the sample extract was poured onto the column cartridge and was allowed to pass through it slowly. The elute was discarded.

Washing of the Column

The column was washed three times with 2 ml deionized water. After the water rinse was complete, the column was allowed to dry for 2 min and a 28 ml glass universal bottle was placed underneath.

Elution

The quinolones were eluted from the cartridge with 2×2 ml of 1% trifluoroacetic acid in acetonitrile followed by 1 ml of acetonitrile.

Drying and reconstitution

The eluent was dried and concentrated on rotary evaporator at 50°C. The residue was reconstituted in the mobile phase of HPLC and was filtered (0.45 μm, 13 mm) prior to analysis on HPLC.

Method Validation:

The next step was testing the optimized procedures under various validation conditions to make sure these are reliable, reproducible and can be used with confidence when applied to real samples. This was done by studying various parameters as listed below.

- Stability studies
    a. Freeze-and-thaw stability
    b. Freezing period stability
    c. Room temperature stability
- Precision
- Accuracy
- Linearity
- Range
- Recovery
- Limit of Detection
- Limit of Quantitation

1. Stability studies

Monitoring of stability of the analytes in samples is crucial as it determines the ultimate fate of the results. A sample is usually carried to laboratory and stored frozen, so both the factors i.e. sample matrix and storage conditions which can affect the analyte should be validated before proceeding towards the sample analysis so that any alteration introduced by these factors may be ruled out (2002/657/EC, 2002; www.fda.gov).

a. Freeze-and-thaw stability

To access the stability of the analytes after freezing-thawing, three blank meat samples were spiked at 50 ng g^{-1}. Then these samples were

subjected to freeze-thaw cycle (frozen for 24 h and then thawed) as all the samples were passed through these phases.

b. Freezing period stability

The effect of freezing duration was studied by spiking blank meat samples at 100 ng g^{-1} in triplicate and then freezing for three weeks as all the samples were collected and stored within this period. Samples were analyzed before freezing, at 24h of freezing and then after three weeks of freezing (Bailac *et al.*, 2006).

c. Stability of samples at room temperature

Three extracts from blank meat samples were spiked with a 100 ng g^{-1} conc. of quinolones and were analyzed immediately (t=0). These were then left at room temperature as such until 24 h at which time these were again analyzed. The difference between the two time points was calculated for each.

2. Linearity and Range

This was done by running various concentrations of the standard solutions of quinolone antibiotics in HPLC and recording the peak areas. The data for peak areas corresponding to each concentration level was used for plotting a curve between these two variables to work out the linearity and range for each analyte. The concentrations used were 200, 150, 100, 80, 60, 40, 20, 10, 5 and 2.5 ng g^{-1} for each of the quinolone.

Stock standard solution:

Stock standard solutions were prepared by dissolving individual standard in 0.01% NaOH solution to increase their solubility. Stock solutions were stored at -4°C (Samanidou *et al.*, 2008; Naeem *et al.*, 2006).

Working standard solutions:

Working standard solutions were prepared from these by dissolving in deionized water (Table-6).

Table-6: Preparation of standard solutions of quinolones

Standard No.	Standard solution added	Water added (ml)	Final conc. (ng ml^{-1})
1	20 ml of 1000 ng ml^{-1} solu.	80	200
2	75 ml of 200 ng ml^{-1} solu.	25	150
3	50 ml of 200 ng ml^{-1} solu.	50	100
4	40 ml of 200 ng ml^{-1} solu.	60	80
5	30 ml of 200 ng ml^{-1} solu.	70	60
6	20 ml of 200 ng ml^{-1} solu.	80	40
7	10 ml of 200 ng ml^{-1} solu.	90	20
8	5 ml of 200 ng ml^{-1} solu.	95	10
9	2.5 ml of 200 ng ml^{-1} solu.	97.5	5
10	1.25 ml of 200 ng ml^{-1} solu.	98.75	2.5

Recovery, Precision and Accuracy

To determine intra-day accuracy and precision of the assay, three standard samples spiked at three concentration levels each (50, 100 and 150 ng g^{-1} for OFL, NOR, CIP, ENR, SAR, OXO and FLU were extracted using the optimized procedure for this purpose and analyzed with HPLC. Three replicates were analyzed in a day (for intra-day precision and accuracy) and the same was done for three different days (inter-day precision and accuracy). Each day, separately weighed stock solutions of the analytes were prepared.

	Day-1	**Day-2**	**Day-3**	
	Replicate I	Replicate I	Replicate I	
	Replicate II	Replicate II	Replicate II	
	Replicate III	Replicate III	Replicate III	
Inter-day	Mean	Mean	Mean	**Inter-day**
	Intra-day	**Intra-day**	**Intra-day**	

LOD and LOQ

Limit of Detection

Limit of detection is the minimum amount of analyte that can be detected by the instrument being used. This was done by running various concentrations of each of the quinolones from high to low and comparing signal-to-noise ratio. LOD is usually a signal-to-noise ratio equal to 3 while the LOQ which is taken much higher than LOD for more accuracy is signal-to-noise ratio of 10 (Bailac *et al.*, 2006).

Phase II

Post-optimization samples analysis

Once the whole method for the extraction and analysis of quinolones from the poultry meat matrix was optimized and validated this wa applied to the real samples to conduct a survey for the violations of quinolones use in broiler poultry farming. This also aimed at posing the level of threat to the consumers as all the samples were collected from the meat shops where meat I available ready for use.

Samples collection/storage:

Meat samples were collected randomly from various poultry shops of Rawalpindi and Islamabad. Samples were brought directly to the National Veterinary Laboratories, Islamabad.

Samples storage:

All the samples were stored in freezer (-20°C) upon arrival until the time of analysis (1-2 weeks).

Samples grouping:

A total of 60 samples were collected. For convenience, all the samples were divided into two groups:

- Those collected from Islamabad (designated as I-1 to I-30)
- Those collected from Rawalpindi (designated as R-1 to R-30)

Samples preparation and analysis

All the samples were processed for extraction and were then analyzed by HPLC using the optimized methods. The concentration of analytes was calculated by the following formula:

$$\text{Analyte conc (ng g}^{-1}) = \frac{\text{AUC sample} \times \text{STD purity}}{\text{AUC standard}} \times 100$$

Where,

AUC=Area under curve
STD=Standard

Secondary confirmation of positive samples

Although HPLC is sensitive enough to confirm the presence of an analyte but for more confirmation a secondary confirmatory checking was applied by FTIR. This was done by dissecting the peaks from HPLC at their specified elution time.

Peak dissection:

Since all the peaks are eluted separately and a t a specific time regarded as retention time, so at this time the mobile phase containing a specific peak was collected close to the detector as soon as it emerges from the detector into clean vials. This was dried under nitrogen flow and reconstituted.

FTIR analysis:

Purging

To minimize any interference from CO_2 or water vapours, the whole chamber of the FTIR spectrophotometer was closed and connected with a cylinder of helium. The chamber was purged with the inert gas and scanned after regular

intervals to confirm the background signals have minimized. Once the chamber was successfully purged, the gas was removed and analysis started.

Scanning

Fourier transform infrared spectrophotometer (FTIR) identification of the eluted peaks from the HPLC was performed as a secondary check and confirmation. For this purpose, a drop of reconstituted sample was placed on the lens and scanning started from the software.

Processing and presentation of results

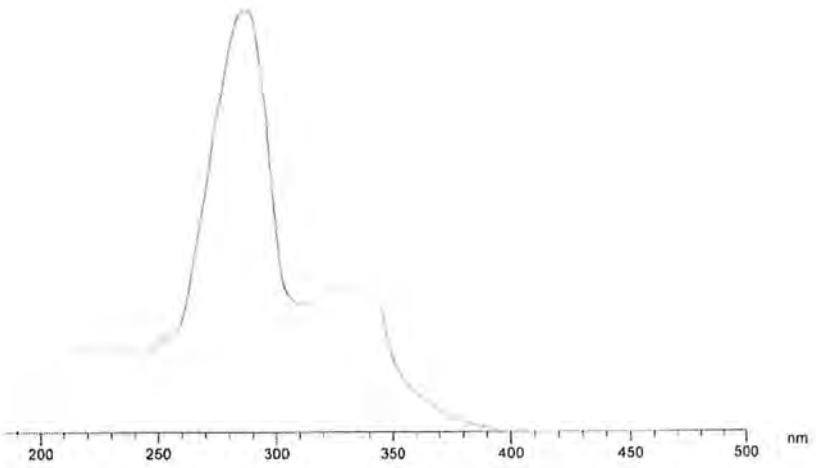
The software automatically compared the results with the built-in library and presented results as percentage of matching with the quinolones spectra to confirm the presence or absence.

For the sake of convenience the results are presented below in two main categories i.e. optimization & validation phase and the second phase which comprised of application to real samples:

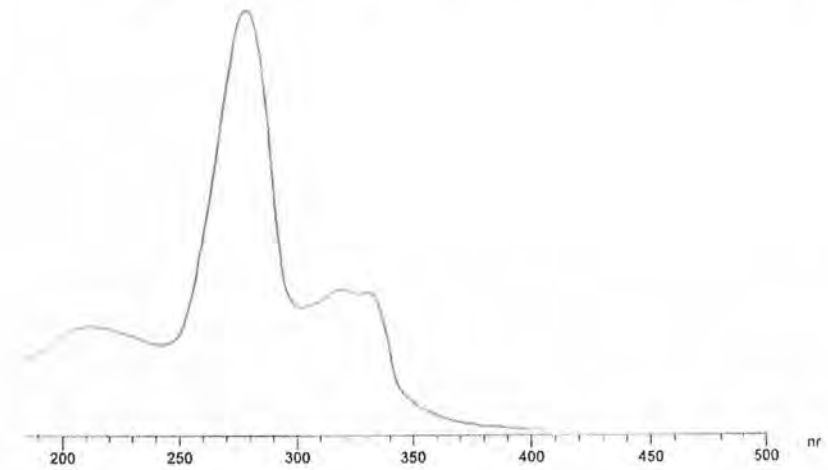
A. Optimization & Validation Phase

UV/Vis scans of Standards

The absorption patter of the antibiotics (OFL, NOR, CIP, EFX, SAR, DIF, OXO and FLU) showed trends towards 280 nm for all except OXO and FLU while lower for the latter. The full range UV/Vis scans are shown in Fig-4 which shows A_{max} for individual antibiotics while Fig-5 shows a comparision of A_{max} for all the antibiotics.

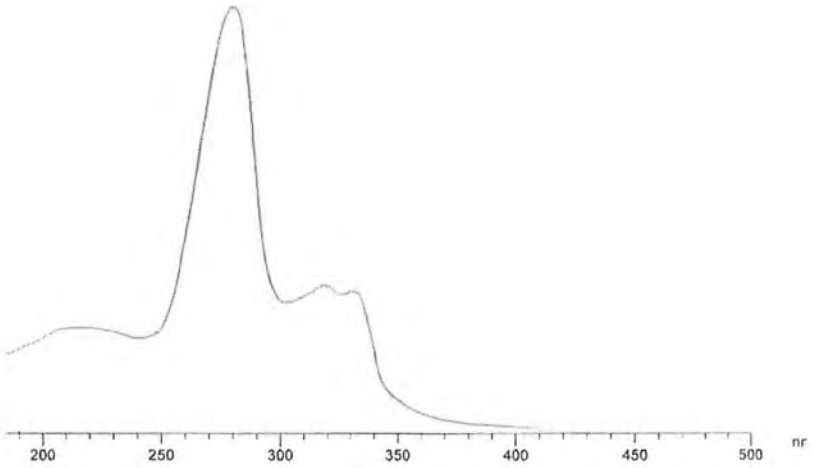


a. Ofloxacin

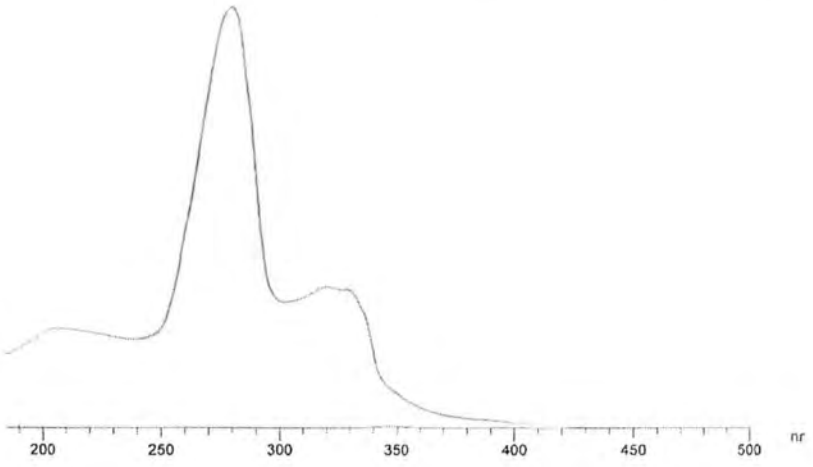


b. Norfloxacin

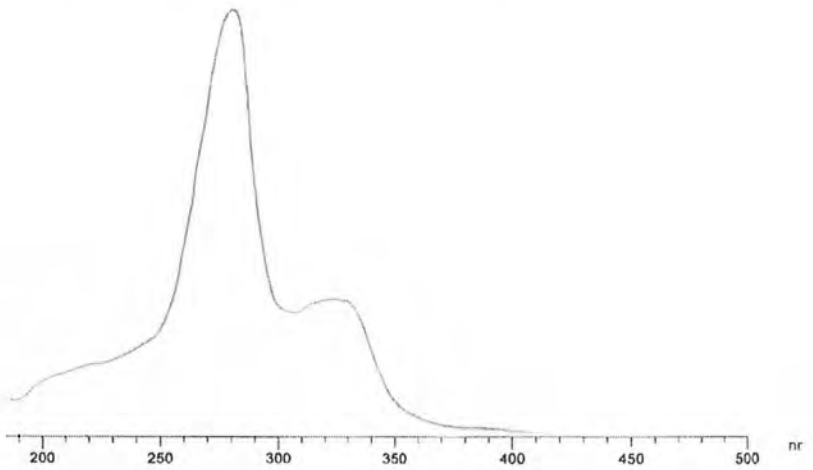
c. Ciprofloxacin



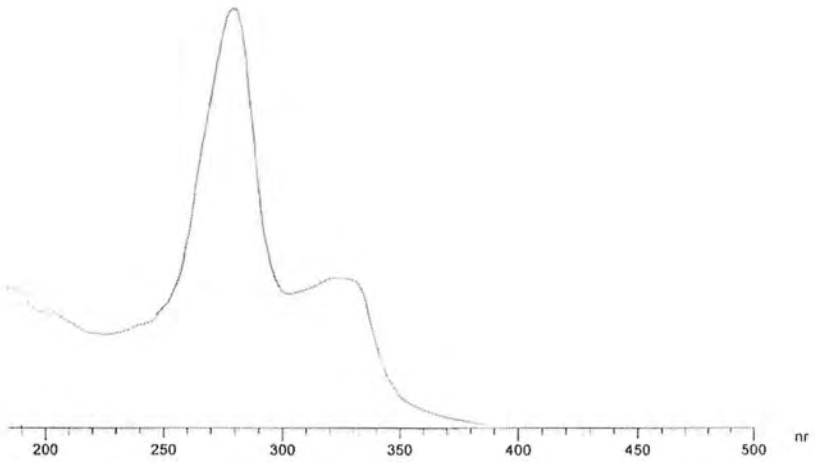
d. Enrofloxacin



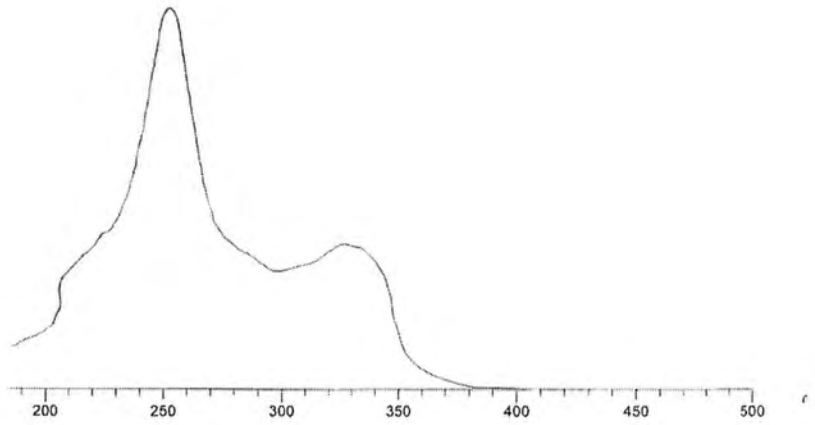
e. Sarafloxacin



f. Difloxacin



g. Oxolinic acid



h. Flumequine

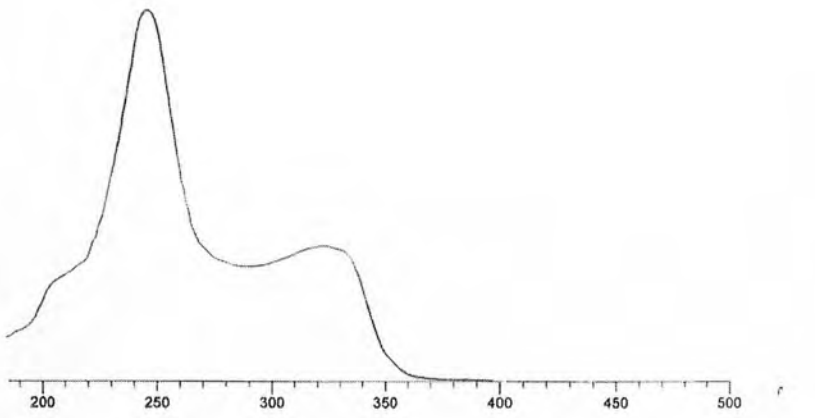


Fig-4: UV/Vis scans of various quinolones studied

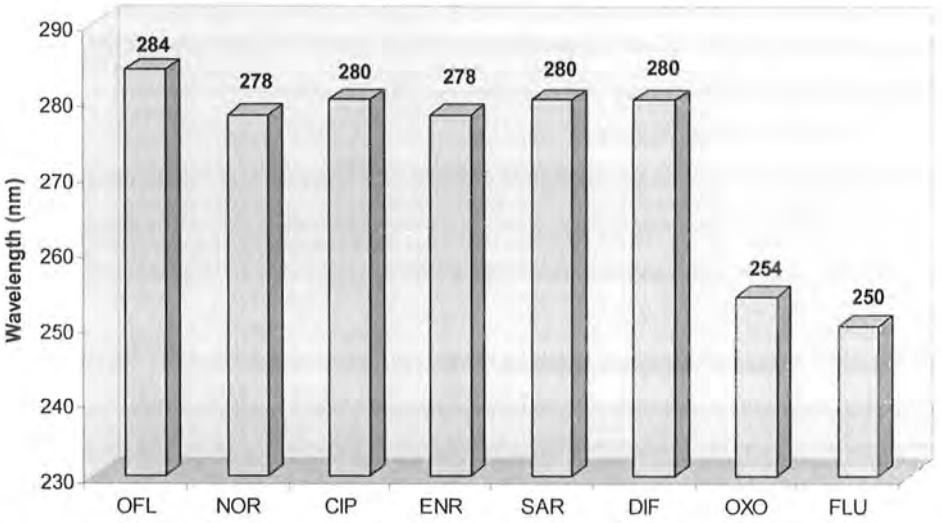


Fig-5: A_{max} of various quinolones scanned with UV/Vis spectrophotometer

Chromatographic conditions

Different combinations of methanol, acetonitrile and buffer (citric acid) were initially tried keeping the buffer volume constant, initially with 10 mM citric acid at pH 3.0. and flow rate of 1.2 ml min^{-1} while the UV detection wavelength was 280 nm for all quinolones except OXO and FLU (252 nm). Good separation was observed with the acetonitrile:methanol:buffer at 10:12:80, except SAR and DIF who had overlapping peaks (Fig-6, 7).

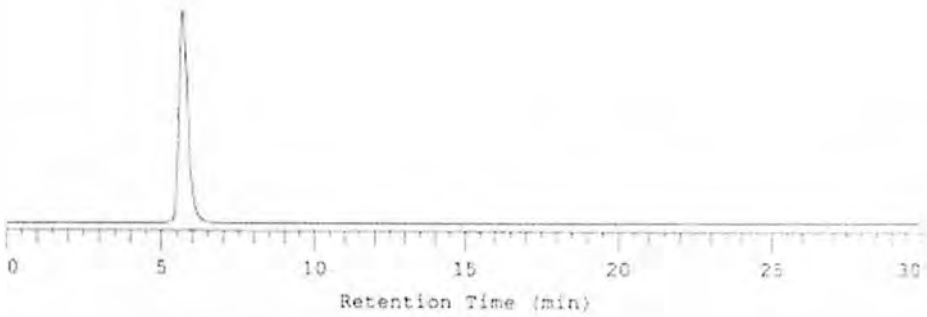


Fig-6 (a). Ofloxacin standard

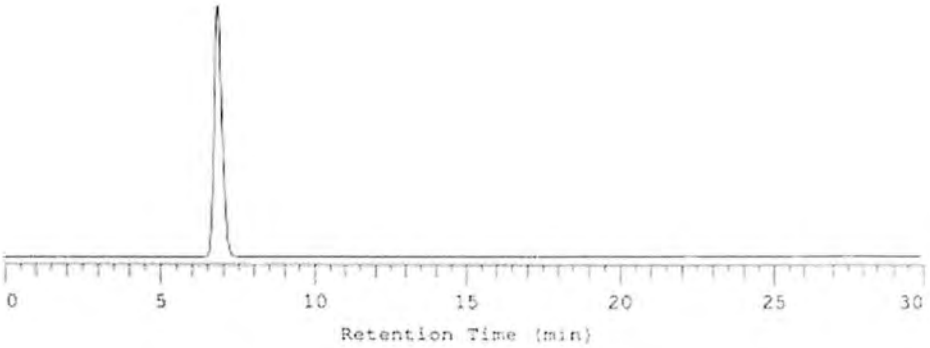


Fig-6 (b). Norfloxacin standard

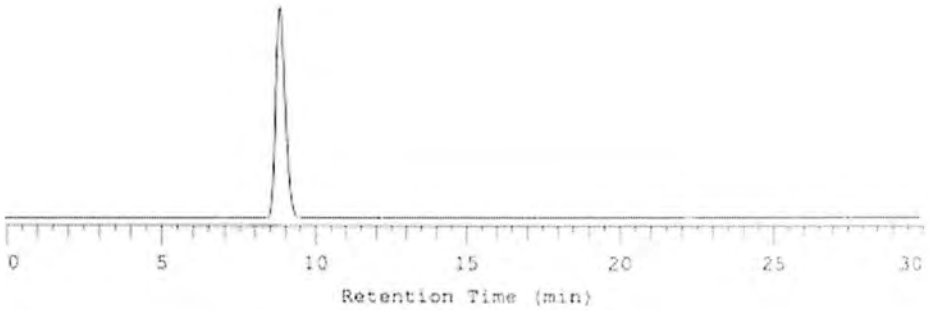


Fig-6 (c). Ciprofloxacin standard

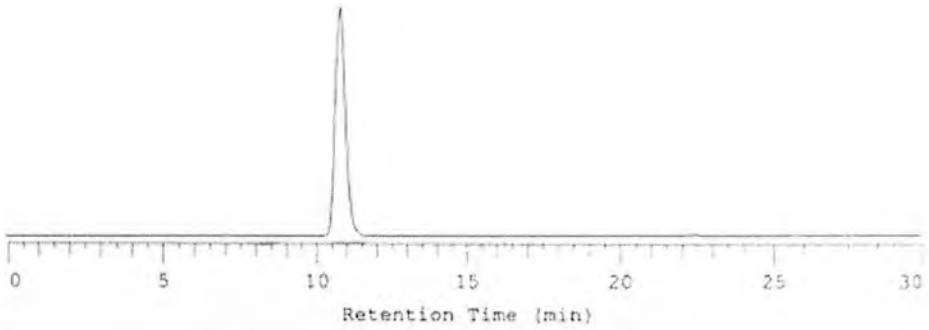


Fig-6 (d). Enrofloxacin standard

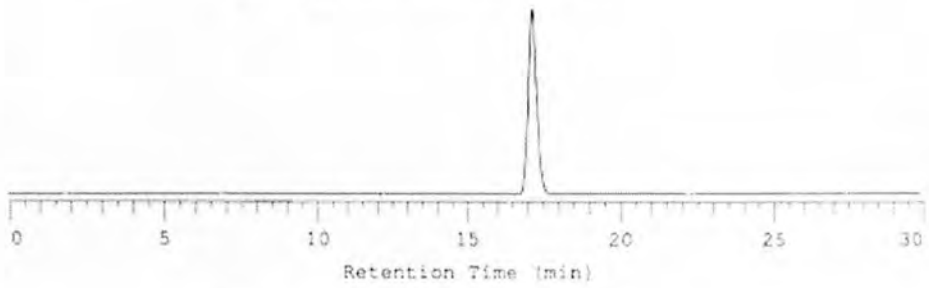


Fig-6 (e). Sarafloxacin standard

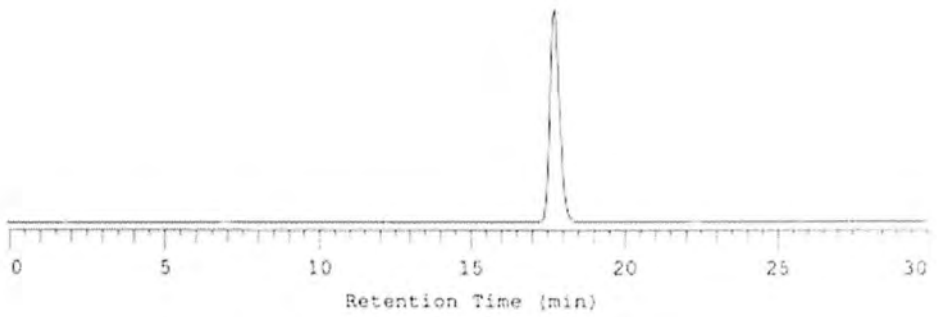


Fig-6 (f). Difloxacin standard

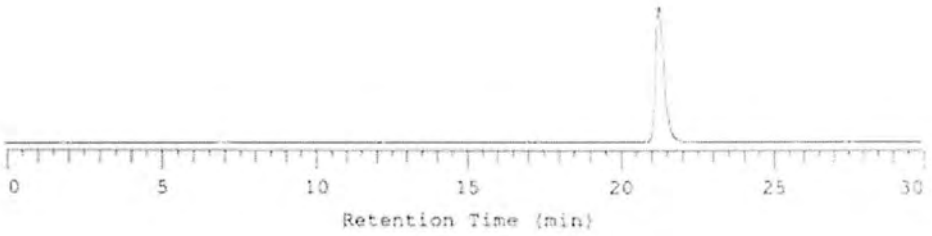


Fig-6 (g). Oxolinic acid standard

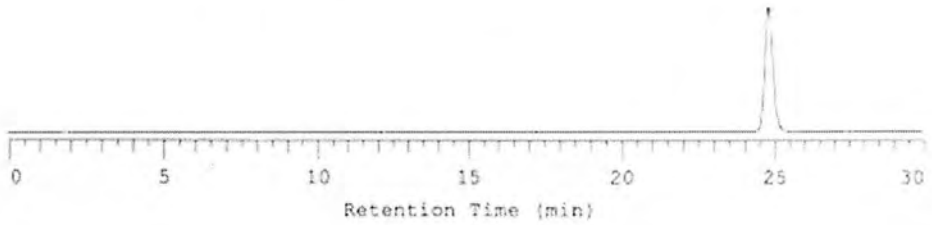


Fig-6 (h). Flumequine standard

Fig-6: HPLC chromatograms of individual quinolones at pH 3.0.

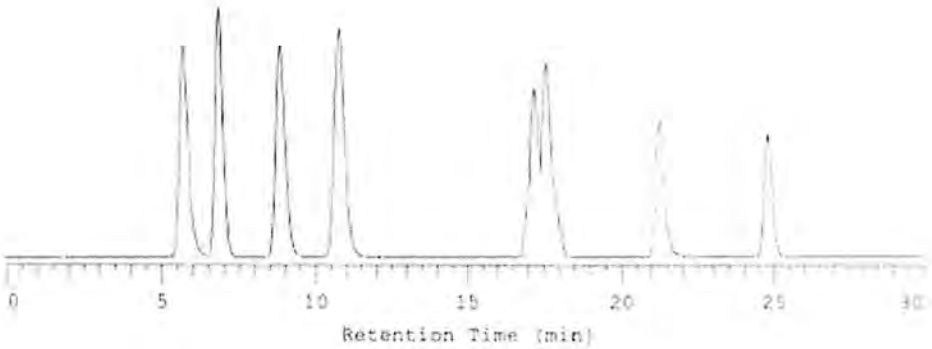


Fig-7: HPLC chromatogram of quinolones at pH 3.0. In order from left to right are ofloxacin, norfloxacin, ciprofloxacin, enrofloxacin, sarafloxacin, difloxacin, oxolinic acid and flumequine standards in a single run.

Buffer pH

The problem of resolution of SAR and DIF was resolved by trying a lower pH (4.5) buffer which separated SAR and DIF (Fig-8). However, this was not a suitable pH for OFL and NOR as they nearly merged. Therefore, it was not possible to separate all the quinolones under study to separate with isocratic conditions so a gradient was required using two buffers at different pH values (pH 3.5 and 4.5).

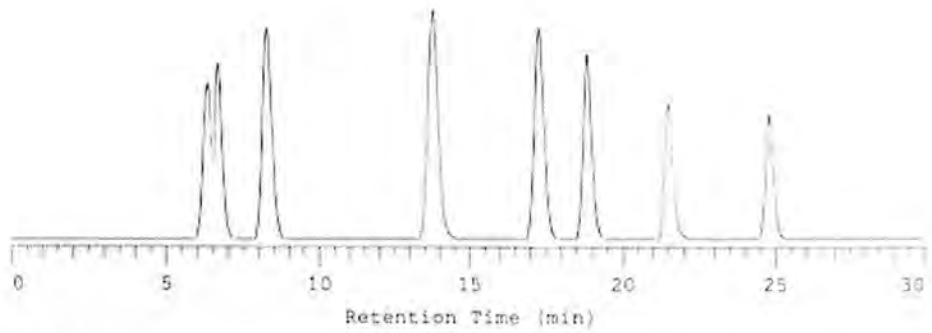


Fig-8: HPLC chromatogram of quinolones at pH 4.5. In order from left to right are ofloxacin, norfloxacin, ciprofloxacin, enrofloxacin, sarafloxacin, difloxacin, oxolinic acid and flumequine standards in a single run.

Samples preparation

The tables 7 and 8 summarize the recoveries of quinolones under study while Fig-9 compares recoveries with both the methods.

Table-7: Percent recovery of various quinolones with method-I using phosphate buffer extraction

Analyte	Spiked conc. ($ng\ g^{-1}$)	Recovered ($ng\ g^{-1}$)	Percent recovery	RSD (%)
OFL	100	43.8±2.81	48.8	5.76
NOR	100	63.7±3.21	63.7	5.03
CIP	100	55.3±2.12	55.3	3.83
ENR	100	68.8±1.73	68.8	2.51
SAR	100	57.7±2.13	57.7	3.69
DIF	100	68.5±1.34	68.5	1.95
OXO	100	60.6±2.21	60.6	3.65
FLU	100	64.1±4.11	64.1	6.41

Table-8: Percent recovery of various quinolones with method-II using trifluoroacetic acid extraction

Analyte	Spiked conc. ($ng\ g^{-1}$)	Recovered ($ng\ g^{-1}$)	Percent recovery	RSD (%)
OFL	100	78.6±2.12	78.6	2.69
NOR	100	88.1±1.34	88.1	1.52
CIP	100	81.6±2.31	81.6	2.83
ENR	100	78.2±2.71	91.1	2.97
SAR	100	87.9±2.15	87.9	2.44
DIF	100	85.3±1.51	85.3	1.77
OXO	100	79.7±2.33	75.7	3.07
FLU	100	82.8±1.92	82.8	2.29

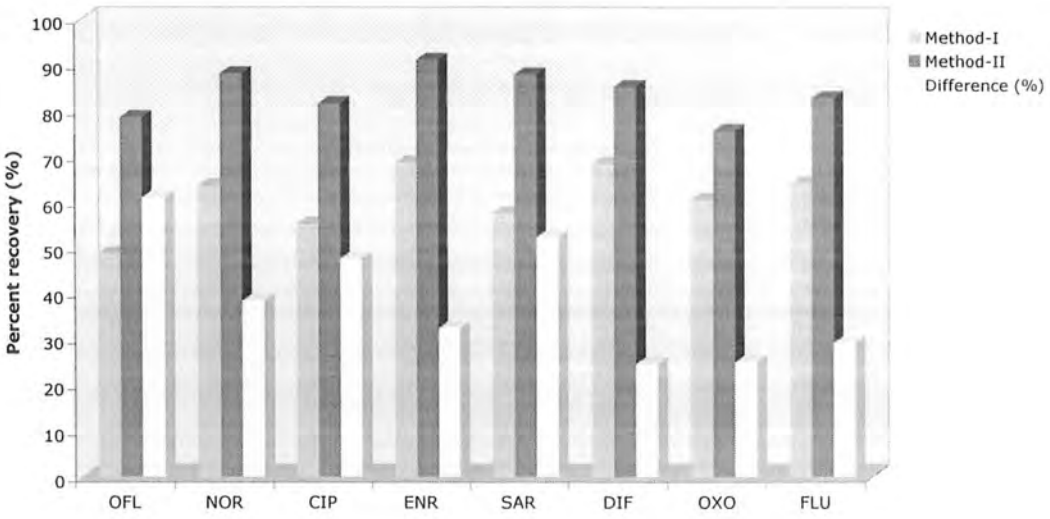


Fig-9: Comparison of the two extraction methods for quinolones

Method validation

- Stability studies
- Precision
- Accuracy
- Linearity
- Range
- Recovery
- Limit of Detection (LOD)
- Limit of Quantitation (LOQ)

Stability studies

Freeze-and-thaw stability

Table-9 shows in comparison the effect of freeze-thaw cycle on recovery of quinolones while Fig-10 shows percent deviation in the recovery of analytes from meat samples after freeze-thaw cycle.

Table-9: Comparison of effect of freeze-thaw cycle on recovery of quinolones

Analyte	Spiked ($ng\ g^{-1}$)	Recovered ($ng\ g^{-1}$)	Expected recovery ($ng\ g^{-1}$)
OFL	100	78.9±2.31	78.6±2.12
NOR	100	87.2±1.12	88.1±1.34
CIP	100	81.2±1.56	81.6±2.31
ENR	100	89.3±2.23	91.1±2.71
SAR	100	86.9±2.61	87.9±2.15
DIF	100	85.1±2.78	85.3±1.51
OXO	100	75.9±2.12	75.7±1.23
FLU	100	83.0±1.56	82.8±1.92

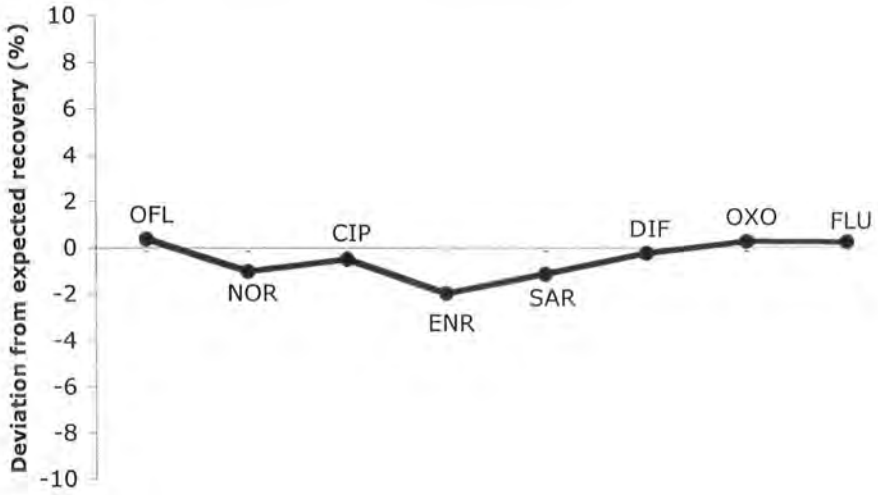


Fig-10: Percent deviation in the recovery of analytes from meat samples after freeze-thaw cycle

Freezing period

Table-10 compares effect of freezing period on recovery of quinolones while Fig-11 shows percent deviation in the recovery of quinolones after three weeks of freezing.

Table-10: Comparison of effect of freezing period on recovery of quinolones

Analyte	Spiked (ng g^{-1})	Recovered (ng g^{-1})	Expected recovery (ng g^{-1})
OFL	100	78.1±1.12	78.6±2.12
NOR	100	88.7±1.56	88.1±1.34
CIP	100	80.2±2.12	81.6±2.31
ENR	100	90.1±2.23	91.1±2.71
SAR	100	85.6±2.61	87.9±2.15
DIF	100	84.8±1.78	85.3±1.51
OXO	100	74.9±1.12	75.7±1.23
FLU	100	83.4±1.56	82.8±1.92

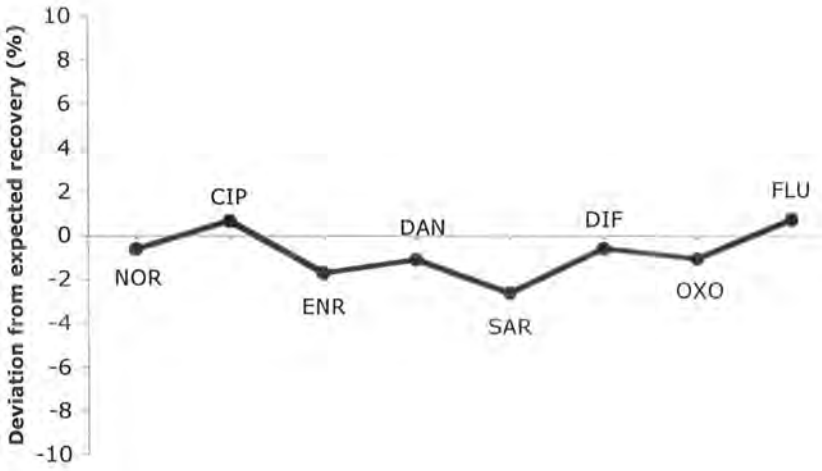


Fig-11: Percent deviation in the recovery of quinolones after three weeks of freezing

Stability of samples at room temperature

Table-11 shows fffect of room temperature on stability of quinolones during a period of 24h while Fig-12 demonstrates effect of room temperature on samples at t=24h compared with t=0

Table-11: Effect of room temperature on stability of quinolones during a period of 24h

Analyte	Spike level ($ng\ g^{-1}$)	Conc. at t=0 ($ng\ g^{-1}$)	Conc. at t=24h ($ng\ g^{-1}$)
OFL	100	99.6±0.65	99.1±0.31
NOR	100	100.1±0.23	100.5±0.27
CIP	100	99.6±0.13	99.2±0.18
ENR	100	98.9±0.15	99.4±0.45
SAR	100	98.6±0.21	97.9±0.26
DIF	100	99.7±0.61	99.6±0.45
OXO	100	100.2±0.48	99.9±0.51
FLU	100	98.5±0.28	97.9±0.47

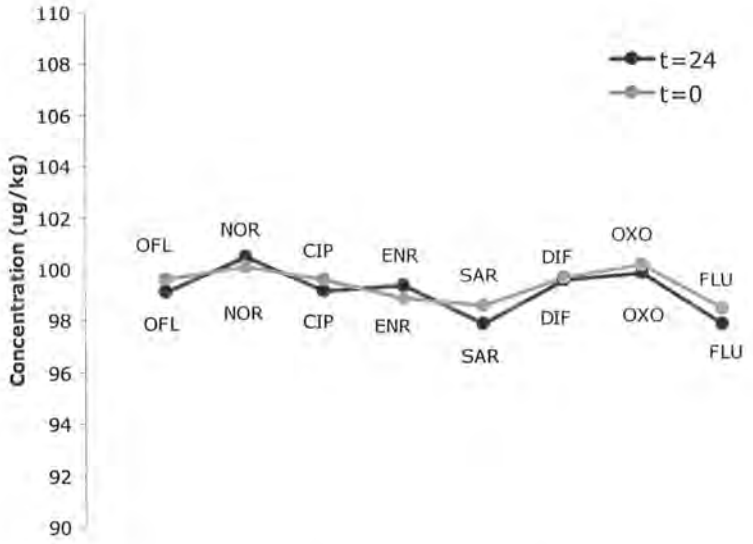


Fig-12: Effect of room temperature on samples at t=24h compared with t=0

Recovery

Precision and accuracy

A good recovery of all the analytes under study was achieved with the extraction and cleanup procedures used (Tables-7 to -16).

Table-12: Inter-day and intra-day precision and accuracy for ofloxacin

Parameter	Spike level (ng g^{-1})	Intraday				Interday (n=9)
		Replicate	Day 1 (n=3)	Day 2 (n=3)	Day 3 (n=3)	
Conc. found (ng g^{-1})	50	I	39.2	38.2	39.8	
		II	38.7	38.1	40.7	
		III	39.7	39.1	39.8	39.26±0.82
		X_{50}	39.20	38.47	40.10	
	100	I	78.6	79.5	77.9	
		II	77.8	80.6	78.2	
		III	79.6	78.2	77.1	78.61±0.85
		X_{100}	78.67	79.43	77.73	
	150	I	119.8	118.8	117.8	
		II	119.9	119.3	118.6	
		III	120.5	120.1	120.5	
		X_{150}	120.07	119.40	118.97	119.70±0.34
Recovery (%)	50	I	78.4	76.4	79.6	
		II	77.4	76.2	81.4	
		III	79.4	78.2	79.6	78.51±1.64
		X_{50}	78.4	76.93	80.2	
	100	I	78.6	79.5	77.9	
		II	77.8	80.6	78.2	
		III	79.6	78.2	77.1	78.61±0.85
		X_{100}	78.67	79.43	77.73	
	150	I	79.87	79.20	79.87	
		II	79.93	79.53	79.07	
		III	80.33	80.07	80.33	
		X_{150}	80.04	79.60	79.76	79.80±0.23
Accuracy (%)	50	X_{50}	78.4	76.93	80.2	78.51±1.64
	100	X_{100}	78.67	79.43	77.73	78.61±0.85
	150	X_{150}	80.04	79.60	79.76	79.80±0.22
Precision (%RSD)	50	X_{50}	1.28	1.43	1.30	2.08
	100	X_{100}	1.15	1.51	0.73	1.08
	150	X_{150}	0.32	0.55	0.80	0.46

Table-13: Inter-day and intra-day precision and accuracy for norfloxacin

Parameter	Spike level (ng g^{-1})	Intraday				Interday (n=9)
		Replicate	Day 1 (n=3)	Day 2 (n=3)	Day 3 (n=3)	
Conc. found (ng g^{-1})	50	I	44.1	43.9	42.7	
		II	44.6	43.2	41.7	
		III	43.7	42.7	42.3	
		X_{50}	44.13	43.27	42.23	43.21±0.95
	100	I	88.6	89.2	86.2	
		II	89.9	88.7	86.5	
		III	89.5	89.2	87.3	
		X_{100}	89.33	89.03	86.67	88.34±1.46
	150	I	134.2	132.7	135.8	
		II	133.9	132.8	135.2	
		III	135.3	131.4	136.2	
		X_{150}	134.47	132.30	135.73	134.17±1.74
Recovery (%)	50	I	88.2	87.8	85.4	
		II	89.2	86.4	83.4	
		III	87.4	85.4	84.6	
		X_{50}	88.27	86.53	84.47	86.42±1.90
	100	I	88.6	89.2	86.2	
		II	89.9	88.7	86.5	
		III	89.5	89.2	87.3	
		X_{100}	89.33	89.03	86.67	88.34±1.46
	150	I	89.47	88.47	90.53	
		II	89.27	88.53	90.13	
		III	90.2	87.6	90.8	
		X_{150}	89.64	88.2	90.49	89.44±1.16
Accuracy (%)	50	X_{50}	88.27	86.53	84.47	86.42±1.90
	100	X_{100}	89.33	89.03	86.67	88.34±1.46
	150	X_{150}	89.64	88.2	90.49	89.44±1.16
Precision (%RSD)	50	X_{50}	1.02	1.39	1.19	2.20
	100	X_{100}	0.75	0.32	0.66	1.65
	150	X_{150}	0.55	0.59	0.37	1.29

Table-14: Inter-day and intra-day precision and accuracy for ciprofloxacin

Parameter	Spike level (ng g^{-1})	Intraday				Interday (n=9)
		Replicate	Day 1 (n=3)	Day 2 (n=3)	Day 3 (n=3)	
Conc. found (ng g^{-1})	50	I	40.9	39.3	38.9	
		II	39.9	39.9	40.1	
		III	41.7	38.5	39.9	
		X_{50}	40.83	39.23	39.63	39.90±0.83
	100	I	82.3	80.4	80.1	
		II	81.6	79.9	79.9	
		III	83.1	81.6	81.4	
		X_{100}	82.33	80.63±	80.47±	81.14±1.03
	150	I	121	122.1	119.8	
		II	122.2	123.5	120.2	
		III	120.6	121.1	118.6	
		X_{150}	121.27	122.23	119.53	121.01±1.37
Recovery (%)	50	I	81.8	78.6	77.8	
		II	79.8	79.8	80.2	
		III	83.4	77	79.8	
		X_{50}	81.67	78.47	79.27	79.8±1.67
	100	I	82.3	80.4	80.1	
		II	81.6	79.9	79.9	
		III	83.1	81.6	81.4	
		X_{100}	82.33	80.63	80.47	81.14±1.03
	150	I	80.67	81.4	79.87	
		II	81.47	82.33	80.13	
		III	80.40	80.73	79.07	
		X_{150}	80.84	81.49	79.69	80.67±0.91
Accuracy (%)	50	X_{50}	81.67	78.47	79.27	79.8±1.67
	100	X_{100}	82.33	80.63	80.47	81.14±1.03
	150	X_{150}	80.84	81.49	79.69	80.67±0.91
Precision (%RSD)	50	X_{50}	2.208612	1.790256	1.622145	2.09
	100	X_{100}	0.911606	1.083534	1.012162	1.27
	150	X_{150}	0.686641	0.986264	0.696598	1.13

Table-15: Inter-day and intra-day precision and accuracy for enrofloxacin

Parameter	Spike level (ng g^{-1})	Intraday				Interday (n=9)
		Replicate	Day 1 (n=3)	Day 2 (n=3)	Day 3 (n=3)	
Conc. found (ng g^{-1})	50	I	44.3	45.1	46.6	
		II	45.2	45.3	46.1	
		III	43.7	43.9	45.7	
		X_{50}	44.40	44.77	46.13	45.10±0.91
	100	I	91.1	92.7	90.1	
		II	91.9	92.9	91.5	
		III	90.2	91.5	89.7	
		X_{100}	91.07	92.37	90.43	91.29±0.99
	150	I	134.6	132.9	133.8	
		II	133.9	132.8	134.2	
		III	135.3	131.4	136.1	
		X_{150}	134.60	132.37	134.70	133.89±1.32
Recovery (%)	50	I	88.6	90.2	93.2	
		II	90.4	90.6	92.2	90.20±1.83
		III	87.4	87.8	91.4	
		X_{50}	88.8	89.53	92.27	
	100	I	91.1	92.7	90.1	
		II	91.9	92.9	91.5	
		III	90.2	91.5	89.7	
		X_{100}	91.07	92.37	90.43	91.29±0.99
	150	I	89.73	88.6	89.2	
		II	89.27	88.53	89.47	
		III	90.2	87.6	90.73	
		X_{150}	89.73	88.24	89.8	89.26±0.88
Accuracy (%)	50	X_{50}	88.8	89.53	92.27	90.20±1.83
	100	X_{100}	91.07	92.37	90.43	91.29±0.99
	150	X_{150}	89.73	88.24	89.8	89.26±0.88
Precision (%RSD)	50	X_{50}	1.70	1.69	0.98	2.03
	100	X_{100}	0.93	0.82	1.05	1.08
	150	X_{150}	0.52	0.63	0.91	0.99

Table-16: Inter-day and intra-day precision and accuracy for sarafloxacin

Parameter	Spike level ($ng\ g^{-1}$)	Intraday				Interday (n=9)
		Replicate	Day 1 (n=3)	Day 2 (n=3)	Day 3 (n=3)	
Conc. found ($ng\ g^{-1}$)	50	I	43.1	44.1	41.1	
		II	45.2	43.2	40.2	
		III	43.7	42.2	42.9	
		X_{50}	44.00	43.17	41.40	42.86±1.33
	100	I	88.1	89.2	84.5	
		II	89.9	88.1	85.7	
		III	89.5	89.9	86.3	
		X_{100}	89.17	89.07	85.50	87.91±2.09
	150	I	132.1	130.2	129.2	
		II	132.1	131.8	130.2	
		III	131.3	131.4	130.2	
		X_{150}	131.83	131.13	129.87	130.94±1.00
Recovery (%)	50	I	86.20	88.20	82.20	
		II	90.40	86.40	80.40	
		III	87.40	84.40	85.80	
		X_{50}	88.00	86.33	82.80	85.71±2.66
	100	I	88.1	89.2	84.5	
		II	89.9	88.1	85.7	
		III	89.5	89.9	86.3	
		X_{100}	89.17	89.07	85.50	87.91±2.09
	150	I	88.07	86.80	86.13	
		II	88.07	87.87	86.80	
		III	87.53	87.60	86.80	
		X_{150}	87.89	87.42	86.58	87.30±0.66
Accuracy (%)	50	X_{50}	88.00	86.33	82.80	85.71±2.66
	100	X_{100}	89.17	89.07	85.50	87.91±2.09
	150	X_{150}	87.89	87.42	86.58	87.30±0.66
Precision (%RSD)	50	X_{50}	2.46	2.20	3.32	3.10
	100	X_{100}	1.06	1.02	1.07	2.38
	150	X_{150}	0.35	0.63	0.44	0.76

Table-17: Inter-day and intra-day precision and accuracy for difloxacin

Parameter	Spike level ($ng\ g^{-1}$)	Intraday				Interday (n=9)
		Replicate	Day 1 (n=3)	Day 2 (n=3)	Day 3 (n=3)	
Conc. found ($ng\ g^{-1}$)	50	I	44.1	43.4	41.5	43.08±1.07
		II	44.6	43.2	41.7	
		III	43.7	42.7	42.8	
		X_{50}	44.13	43.10	42.00	
	100	I	86.6	83.2	85.2	85.24±1.61
		II	86.9	84.1	86.5	
		III	85.5	82.9	86.3	
		X_{100}	86.33	83.40	86.00	
	150	I	129.2	132.2	129.8	130.87±1.31
		II	129.4	133.1	132.8	
		III	129.6	130.5	131.2	
		X_{150}	129.40	131.93	131.27	
Recovery (%)	50	I	88.2	86.8	83	
		II	89.2	86.4	83.4	
		III	87.4	85.4	85.6	
		X_{50}	88.27	86.2	84	86.16±2.13
	100	I	86.6	83.2	85.2	
		II	86.9	84.1	86.5	
		III	85.5	82.9	86.3	
		X_{100}	86.33	83.40	86.00	85.24±1.61
	150	I	86.13	88.13	86.53	
		II	86.27	88.73	88.53	
		III	86.40	87.00	87.47	
		X_{150}	86.27	87.96	87.51	87.24±0.88
Accuracy (%)	50	X_{50}	88.27	86.2	84	86.16±2.13
	100	X_{100}	86.33	83.40	86.00	85.24±1.61
	150	X_{150}	86.27	87.96	87.51	87.24±0.88
Precision (%RSD)	50	X_{50}	1.02	0.84	1.67	2.48
	100	X_{100}	0.85	0.75	0.81	1.88
	150	X_{150}	0.15	1.00	1.14	1.00

Table-18: Inter-day and intra-day precision and accuracy for oxolinic acid

Parameter	Spike level ($ng\ g^{-1}$)	Intraday				Interday (n=9)
		Replicate	Day 1 (n=3)	Day 2 (n=3)	Day 3 (n=3)	
Conc. found ($ng\ g^{-1}$)	50	I	39.3	40.5	38.1	
		II	39.1	40.2	37.9	
		III	40.1	39.5	39.1	
		X_{50}	39.50	40.07	38.37	39.31±0.87
	100	I	76.4	76.3	74.4	
		II	76.1	75.2	74.1	
		III	77.2	76.5	74.7	
		X_{100}	76.57	76.00	74.40	75.66±1.12
	150	I	115.1	114.4	113.6	
		II	115	113.9	113.3	
		III	116.2	115.1	114.4	
		X_{150}	115.43	114.47	113.77	114.56±0.84
Recovery (%)	50	I	78.6	81	76.2	
		II	78.2	80.4	75.8	
		III	80.2	79	78.2	
		X_{50}	79	80.13	76.73	78.62±1.73
	100	I	76.4	76.3	74.4	
		II	76.1	75.2	74.1	
		III	77.2	76.5	74.7	
		X_{100}	76.57	76.00	74.40	75.66±1.12
	150	I	76.73	76.27	75.73	
		II	76.67	75.93	75.53	
		III	77.47	76.73	76.27	
		X_{150}	76.96	76.31	75.84	76.37±0.56
Accuracy (%)	50	X_{50}	79	80.13	76.73	78.62±1.73
	100	X_{100}	76.57	76.00	74.40	75.66±1.12
	150	X_{150}	76.96	76.31	75.84	76.37±0.56
Precision (%RSD)	50	X_{50}	1.34	1.28	1.68	2.20
	100	X_{100}	0.74	0.92	0.40	1.49
	150	X_{150}	0.58	0.53	0.50	0.73

Table-19: Inter-day and intra-day precision and accuracy for flumequine

Parameter	Spike level ($ng\ g^{-1}$)	Intraday				Interday (n=9)
		Replicate	Day 1 (n=3)	Day 2 (n=3)	Day 3 (n=3)	
Conc. found ($ng\ g^{-1}$)	50	I	42.1	40.8	39.3	
		II	40.9	41.5	37.6	
		III	43.2	42.2	38.8	
		X_{50}	42.07	41.50	38.57	40.71±1.88
	100	I	82.7	81.2	85.2	
		II	83.9	80.7	84.5	
		III	81.5	82.2	83.3	
		X_{100}	82.70	81.37	84.33	82.80±1.49
	150	I	119.6	120.8	122.1	
		II	118.7	118.9	121.8	
		III	121.1	121.1	123.2	
		X_{150}	119.80	120.27	122.37	120.81±1.37
Recovery (%)	50	I	84.2	81.6	78.6	
		II	81.8	83	75.2	
		III	86.4	84.4	77.6	
		X_{50}	84.13	83	77.13	81.42±3.76
	100	I	82.7	81.2	85.2	
		II	83.9	80.7	84.5	
		III	81.5	82.2	83.3	
		X_{100}	82.70	81.37	84.33	82.80±1.49
	150	I	79.73	80.53	81.40	
		II	79.13	79.27	81.20	
		III	80.73	80.73	82.13	
		X_{150}	79.87	80.18	81.58	80.54±0.91
Accuracy (%)	50	X_{50}	84.13	83	77.13	81.42±3.76
	100	X_{100}	82.70	81.37	84.33	82.80±1.49
	150	X_{150}	79.87	80.18	81.58	80.54±0.91
Precision (%RSD)	50	X_{50}	2.73	1.69	2.27	4.61
	100	X_{100}	1.45	0.94	1.14	1.79
	150	X_{150}	1.01	0.99	0.60	1.13

Table-20: Overall intra-day and inter-day recovery/precision of quinolones

Analyte	Intra-day recovery			Intra-day
	Day-1	Day-2	Day-3	
OFL	79.04±0.88	78.65±1.49	79.23±	78.97±1.32
NOR	89.08±0.72	87.92±1.27	87.21±	88.07±3.05
CIP	81.61±0.75	80.20±1.56	79.81±	80.54±0.61
ENR	89.87±1.14	90.05±2.11	90.83±	90.25±1.28
SAR	88.35±0.71	87.61±1.38	84.96±	86.97±1.95
DIF	86.96±1.14	85.85±2.30	85.84±	86.22±1.76
OXO	77.51±1.31	77.48±2.30	75.66±	76.88±1.18
FLU	82.23±2.17	81.52±1.42	81.01±	81.59±3.63

Table-21: Overall intra-day and inter-day precision of quinolones

Analyte	Intra-day recovery			Intra-day
	Day-1	Day-2	Day-3	
OFL	0.92±0.52	1.16±0.53	0.94±0.31	1.01±0.14
NOR	0.77±0.24	0.77±0.56	0.74±0.42	0.76±0.02
CIP	1.27±0.82	1.29±0.44	1.11±0.47	1.22±0.10
ENR	1.05±0.60	1.05±0.57	0.98±0.07	1.03±0.04
SAR	1.29±1.07	1.28±0.82	1.61±1.51	1.39±0.19
DIF	0.67±0.46	0.86±0.13	1.21±0.43	0.91±0.27
OXO	0.89±0.40	0.91±0.38	0.86±0.71	0.89±0.03
FLU	1.73±0.89	1.21±0.42	1.34±0.85	1.42±0.27

Linearity and Range

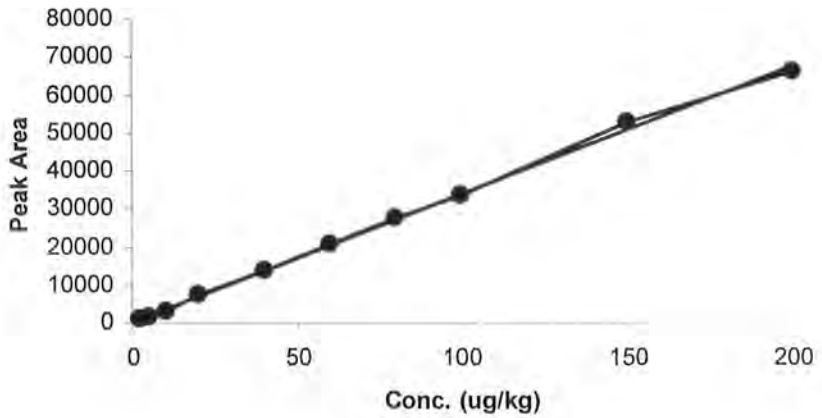
Table-22 shows concentration vs peak areas of various quinolones. The correlation coefficient was between 0.9989 and 0.9997, slope ranged between 162.6 and 392.5 while the intercept was -685.7 to 471.9 (Table-23). While Fig-13 demonstrates calibration curves of quinolones standards studied.

Table-22: Concentration vs peak areas of various quinolones

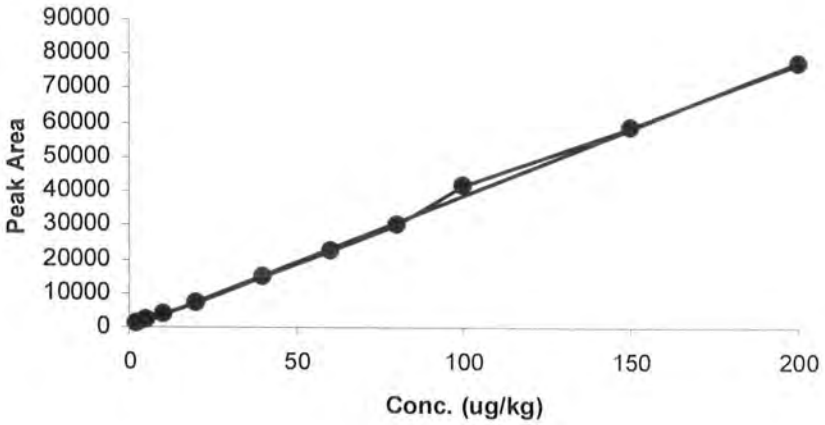
Conc. (ng g^{-1})	OFL	NOR	CIP	ENR	SAR	DIF	OXO	FLU
200	66275	77360	71560	74581	59235	69500	38000	32000
150	53020	58793	50807	58173	45018	53515	29640	24960
100	33642	41591	36141	40314	27810	34577	19487	16410
80	27836	30170	27193	30578	22509	28495	15580	13120
60	20612	22612	20824	22076	16941	21476	12122	10208
40	13849	14646	13596	14916	11255	14248	7458	6281
20	7370	7349	6584	7533	5213	7506	3458	2912
10	3181	3945	4007	3580	3436	3545	1976	1664
5	1581	1944	2004	1780	1701	1763	978	824
2.5	779	967	1017	895	842	891	494	416

Table-23: Characteristics of calibration curves of various quinolones studied

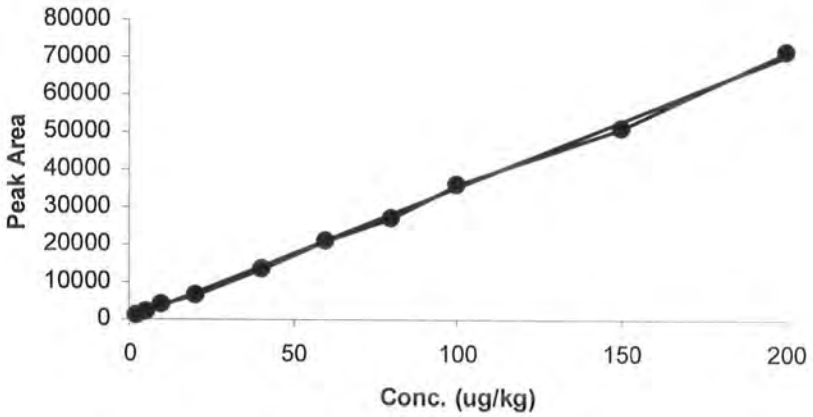
Antibiotic	Slope	Intercept	r^2	LOD	LOQ
OFL	336.3	471.9	0.9989	4.74	15.81
NOR	392.5	-323.6	0.9993	3.85	12.86
CIP	353.3	-313.3	0.9990	3.74	12.47
ENR	380.0	116.0	0.9989	4.21	14.04
SAR	298.3	-685.7	0.9991	4.40	14.69
DIF	348.1	382.9	0.9997	4.37	14.59
OXO	192.9	43.4	0.9993	7.66	25.55
FLU	162.6	25.85	0.9995	9.10	30.34



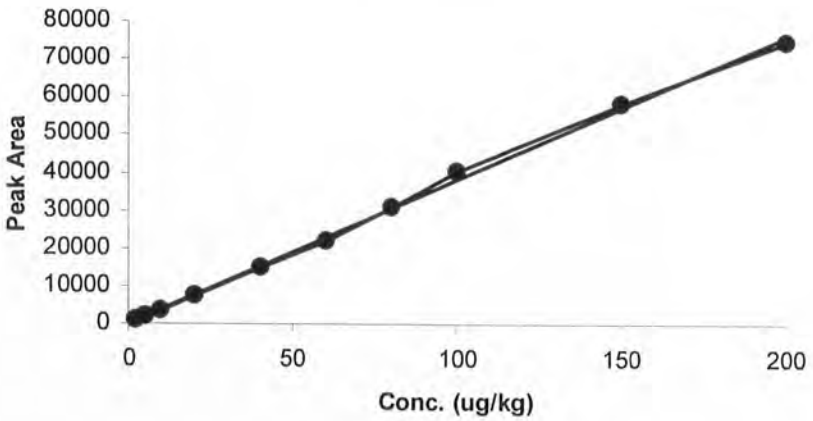
a. Ofloxacin



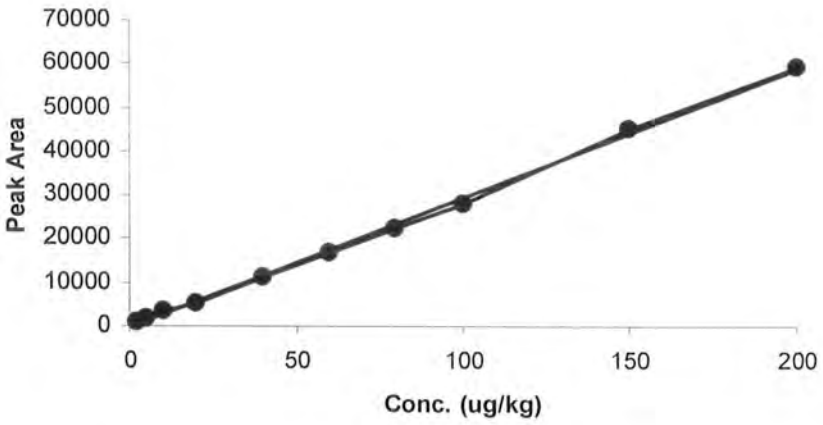
b. Norfloxacin



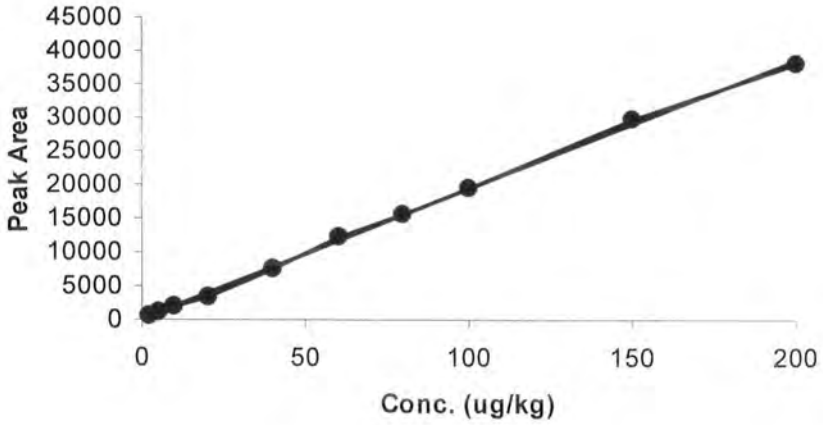
c. Ciprofloxacin



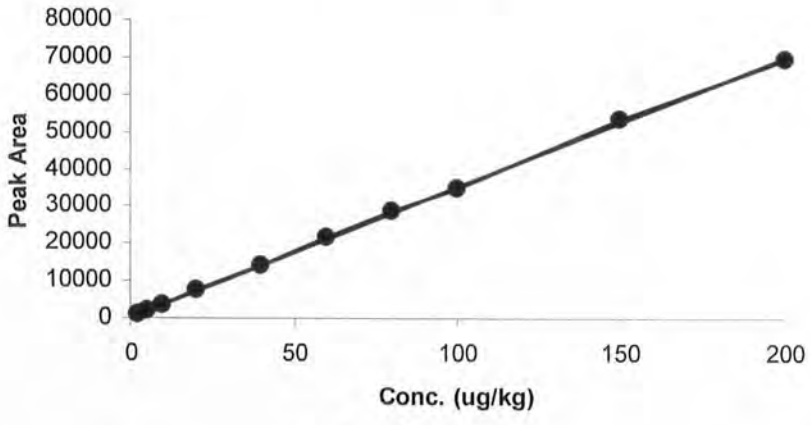
d. Enrofloxacin



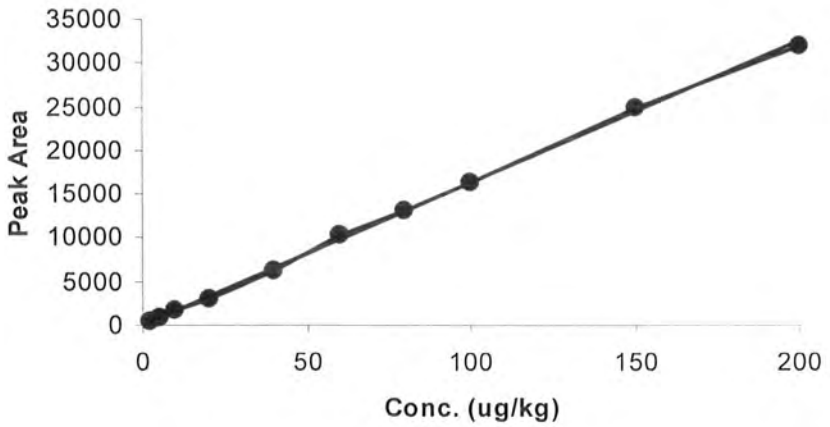
e. Sarafloxacin



f. Difloxacin



g. Oxolinic acid



h. Flumequine

Fig-13: Calibration curves of quinolones standards studied

LOD and LOQ

A good LOD and LOQ were achieved (Fig-14).

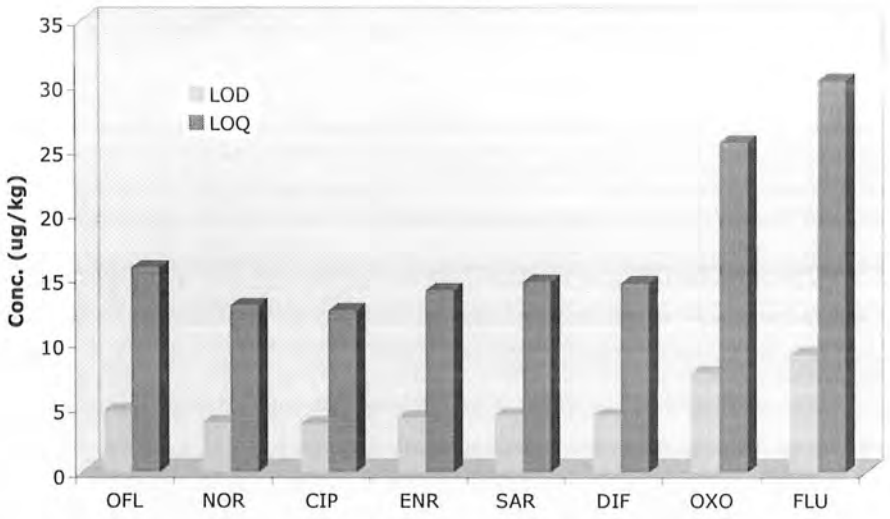


Fig-14: comparison of LOD and LOQ of various quinolones studied

B. Application to Real Samples

Tables-24 to -32 and Fig-15 to -22 show levels of various quinolones in poultry meat samples from Rawalpindi/Islamabad in details.

Table-24: Levels of ofloxacin in meat samples

Sample collection source	Sample No.	Conc. detected (ng g^{-1})	Deviation from LOQ[a] (times LOQ)	Deviation from MRL[b] (times MRL)	Recommended for human consumption/ export[c]?
Islamabad	I-5	7	0.44	0.14	Yes
	I-7	9	0.57	0.18	Yes
	I-16	110	7.00	2.20	No
	I-22	23	1.45	0.46	Yes
Rawalpindi	R-3	110	7.00	2.20	No
	R-8	12	0.76	0.24	Yes
	R-19	12	0.76	0.24	Yes

a=LOQ for ofloxacin was calculated to be 15.81 ng g^{-1}
b=MRL for ofloxacin in chicken not set yet by EU, 50 ng g^{-1} according to The Positive List System of Japan [www.tbt-sps.gov.cn]
c=With respect to **ofloxacin** only, must comply with other requirements as well

Table-25: Levels of norfloxacin in meat samples

Sample collection source	Sample No.	Concentration detected (ng g^{-1})	Deviation from LOQ[a] (times LOQ)	Deviation from MRL[b] (times MRL)	Recommended for human consumption/ export[c]?
Islamabad	I-1	63	4.90	0.63	Yes
	I-5	450	35.0	4.50	No
	I-8	221	17.19	2.21	No
	I-12	89	6.92	0.89	Yes
	I-16	72	5.60	0.72	Yes
	I-19	690	53.7	6.90	No
	I-22	40	3.11	0.40	Yes
	I-24	132	10.26	1.32	No
	I-26	520	40.40	5.20	No
	I-29	790	61.4	7.90	No
	I-30	259	20.14	2.59	No
Rawalpindi	R-1	600	46.60	6.00	No
	R-2	20	1.56	0.20	Yes
	R-6	272	21.15	2.72	No
	R-7	363	28.23	3.63	No
	R-13	245	19.05	2.45	No
	R-16	157	12.21	1.57	No
	R-20	297	23.09	2.97	No
	R-21	840	65.30	8.40	No
	R-24	147	11.43	1.47	No
	R-28	241	18.74	2.41	No
	R-30	128	9.95	1.28	No

a=LOQ for norfloxacin was calculated to be 12.86 ng g^{-1}

b=MRL for norfloxacin in chicken not set yet [www.codexalimentarius.net] so MRL for CIP+ENR=100 ng g^{-1} is considered here

c=With respect to flumequine only, must comply with other requirements as well

Table-26: Levels of ciprofloxacin in contaminated meat samples

Sample collection source	Sample No.	Concentration detected (ng g^{-1})	Deviation from LOQ[a] (times LOQ)	Deviation from MRL[b] (times MRL)
Islamabad	I-1	51	4.09	N/A
	I-3	89	7.14	N/A
	I-5	310	24.9	N/A
	I-9	45	3.61	N/A
	I-11	113	9.06	N/A
	I-13	81	6.50	N/A
	I-15	189	15.16	N/A
	I-17	118	9.46	N/A
	I-19	103	8.26	N/A
	I-20	103	8.26	N/A
	I-23	103	8.26	N/A
	I-25	290	23.3	N/A
	I-26	41	3.29	N/A
	I-27	300	24.1	N/A
	I-28	31	2.49	N/A
Rawalpindi	R-1	69	5.53	N/A
	R-2	21	1.68	N/A
	R-3	114	9.14	N/A
	R-4	201	16.12	N/A
	R-6	370	29.7	N/A
	R-9	66	5.29	N/A
	R-11	51	4.09	N/A
	R-13	111	8.90	N/A
	R-16	370	29.7	N/A
	R-21	51	4.09	N/A
	R-23	138	11.07	N/A
	R-24	151	12.11	N/A
	R-26	81	6.50	N/A
	R-29	59	4.73	N/A

a=LOQ for **ciprofloxacin** was calculated to be 12.47 ng g^{-1}
b=MRL for **ciprofloxacin is** set in combination with enrofloxacin at 100 ng g^{-1}
[www.codexalimentarius.net]

The United States Food and Drug Administration (FDA) has its own regulation regarding use of ENR in food animals and it dose not recommend use this quinolone antibiotic in poultry (www.fda.gov).

Table-27: Levels of enrofloxacin in contaminated meat samples

Sample collection source	Sample No.	Concentration detected (ng g^{-1})±SD	Deviation from LOQ[a] (times LOQ)	Deviation from MRL[b] (times MRL)
Islamabad	I-1	62	4.42	N/A
	I-3	119	8.48	N/A
	I-4	210	15.0	N/A
	I-5	61	4.34	N/A
	I-7	270	19.2	N/A
	I-9	67	4.77	N/A
	I-11	151	10.75	N/A
	I-13	100	7.12	N/A
	I-15	105	7.48	N/A
	I-17	71	5.06	N/A
	I-19	151	10.75	N/A
	I-20	171	12.18	N/A
	I-23	98	6.98	N/A
	I-25	61	4.34	N/A
	I-26	50	3.56	N/A
	I-27	67	4.77	N/A
	I-28	49	3.49	N/A
Rawalpindi	R-1	71	5.06	N/A
	R-2	85	6.05	N/A
	R-3	189	13.46	N/A
	R-4	150	10.68	N/A
	R-6	210	15.0	N/A
	R-9	85	6.05	N/A
	R-11	97	6.91	N/A
	R-13	108	7.69	N/A
	R-16	410	29.2	N/A
	R-18	210	15.0	N/A
	R-21	109	7.76	N/A
	R-23	201	14.32	N/A
	R-24	167	11.89	N/A
	R-26	97	6.91	N/A
	R-29	67	4.77	N/A

a=LOQ for **enrofloxacin** was calculated to be 14.04 ng g^{-1}
b=MRL for **enrofloxacin** is set in combination with ciprofloxacin at 100 ng g^{-1}
[www.codexalimentarius.net]

Table-28: Levels of ciprofloxacin + enrofloxacin in contaminated meat samples

Sample No.	CIP (ng g^{-1})	ENR (ng g^{-1})	CIP+ENR	Deviation from LOQ[a] (times LOQ)	Deviation from MRL[b] (times MRL)	Recommended for human consumption/ export[c]?
I-1	51	62	113	N/A	1.13	No
I-3	89	119	208	N/A	2.08	No
I-4	ND	210	210	N/A	0.21	Yes
I-5	310	61	371	N/A	3.71	No
I-7	ND	270	270	N/A	0.27	Yes
I-9	45	67	112	N/A	1.12	No
I-11	113	151	264	N/A	2.64	No
I-13	81	100	181	N/A	1.81	No
I-15	189	105	294	N/A	2.94	No
I-17	118	71	189	N/A	1.89	No
I-19	103	151	254	N/A	2.54	No
I-20	103	171	274	N/A	2.74	No
I-23	103	98	201	N/A	2.01	No
I-25	290	61	351	N/A	2.90	No
I-26	41	50	91	N/A	0.91	Yes
I-27	300	67	367	N/A	3.67	No
I-28	31	49	80	N/A	0.80	Yes
R-1	69	71	140	N/A	1.40	No
R-2	21	85	106	N/A	1.06	No
R-3	114	189	303	N/A	3.03	No
R-4	201	150	351	N/A	3.51	No
R-6	370	210	580	N/A	5.80	No
R-9	66	85	151	N/A	1.51	No
R-11	51	97	148	N/A	1.48	No
R-13	111	108	219	N/A	2.19	No
R-16	370	410	780	N/A	7.80	No
R-18	ND	21	21	N/A	0.21	Yes
R-21	51	109	160	N/A	1.60	No
R-23	138	201	339	N/A	3.39	No
R-24	151	167	318	N/A	3.18	No
R-26	81	97	178	N/A	1.78	No
R-29	59	67	126	N/A	1.26	No

a=MRL for **ciprofloxacin is** set in combination with enrofloxacin at 100 ng g^{-1}
b=MRL for **enrofloxacin** is set in combination with ciprofloxacin at 100 ng g^{-1} www.codexalimentarius.net]
c=With respect to ciprofloxacin and enrofloxacin residues only, must comply with other requirements as well

Table-29: Levels of sarafloxacin in contaminated meat samples

Sample collection source	Sample No.	Concentration detected (ng g^{-1})	Deviation from LOQ[a] (times LOQ)	Deviation from MRL[b] (times MRL)	Recommended for human consumption/ export[c]?
Islamabad	I-2	8	0.54	0.80	Yes
	I-6	22	1.50	2.20	Yes
	I-22	12	0.82	1.20	Yes
	I-24	9	0.61	0.90	Yes
Rawalpindi	R-15	78	5.31	2.60	No
	R-21	7	0.48	0.70	Yes

a=LOQ for **sarafloxacin** was calculated to be 14.69 ng g^{-1}
b=MRL for **sarafloxacin** set at 30 ng g^{-1} (Samanidou *et al.*, 2008)
c=With respect to flumequine residues only, must comply with other requirements as well

Table-30: Levels of difloxacin in contaminated meat samples

Sample source	Sample No.	Concentration detected (ng g^{-1})±SD	Deviation from LOQ[a] (times LOQ)	Deviation from MRL[b] (times MRL)	Recommended for human consumption/ export[c]?
Islamabad	I-10	19	1.30	0.06	Yes
	I-14	45	3.08	0.15	Yes
Rawalpindi	R-5	10	0.69	0.03	Yes
	R-14	11	0.75	0.04	Yes
	R-22	32	2.19	0.11	Yes

a=LOQ for difloxacin was calculated to be 14.59 ng g^{-1}
b=MRL for difloxacin set at 300 ng g^{-1} [www.codexalimentarius.net]
c=With respect to flumequine only, must comply with other requirements as well

Table-31: Levels of oxolinic acid in contaminated meat samples

Sample collection source	Sample No.	Concentration detected (ng g^{-1})±SD	Deviation from LOQ[a] (times LOQ)	Deviation from MRL[b] (times MRL)	Recommended for human consumption/ export[c]?
Islamabad	I-18	17	0.67	0.17	Yes
Rawalpindi	R-5	110	4.3	1.1	No
	R-11	120	4.7	1.2	No
	R-17	17	0.67	0.17	Yes

a=LOQ for oxolinic acid was calculated to be 25.55 ng g^{-1}
b=MRL for oxolinic acid set at 100 ng g^{-1} [www.codexalimentarius.net]
c=With respect to flumequine only, must comply with other requirements as well

Table-32: Levels of flumequine in contaminated meat samples

Sample collection source	Sample No.	Concentration detected (ng g^{-1})	Deviation from LOQ[a] (times LOQ)	Deviation from MRL[b] (times MRL)	Recommended for human consumption/ export[c]?
Islamabad	I-11	19	0.63	0.04	Yes
	I-21	110	3.63	0.22	Yes
	I-23	510	16.8	1.0	No
	I-27	25	0.82	0.05	Yes
Rawalpindi	R-5	24	0.79	0.05	Yes
	R-10	610	20.1	1.2	No
	R-19	750	24.7	1.5	No
	R-25	13	0.43	0.03	Yes
	R-27	32	1.05	0.06	Yes

a=LOQ for flumequine was calculated to be 30.34 ng g^{-1}
b=MRL for flumequine set at 500 ng g^{-1} [www.codexalimentarius.net]
c=With respect to flumequine only, must comply with other requirements as well

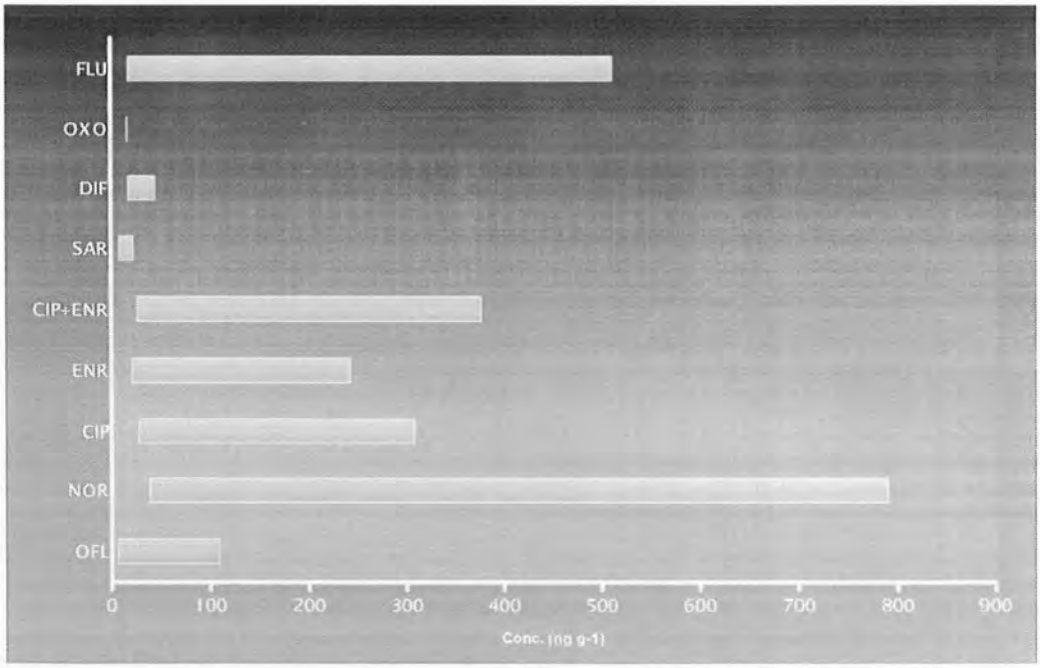


Fig-15: Range of quinolones in contaminated meat samples from Islamabad

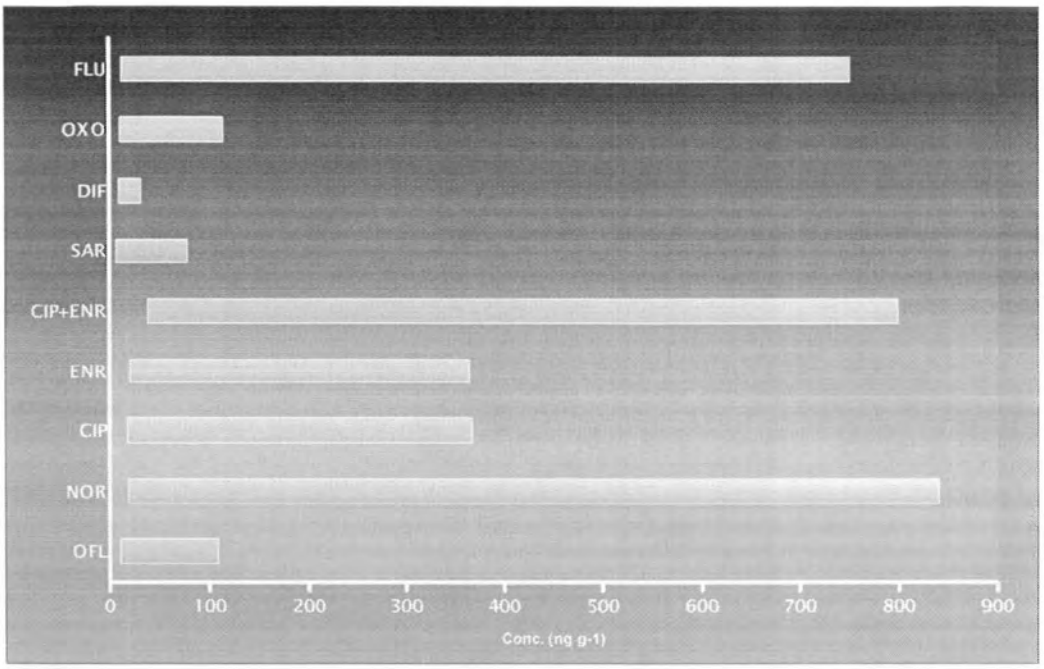


Fig-16: Range of quinolones in contaminated meat samples from Rawalpindi

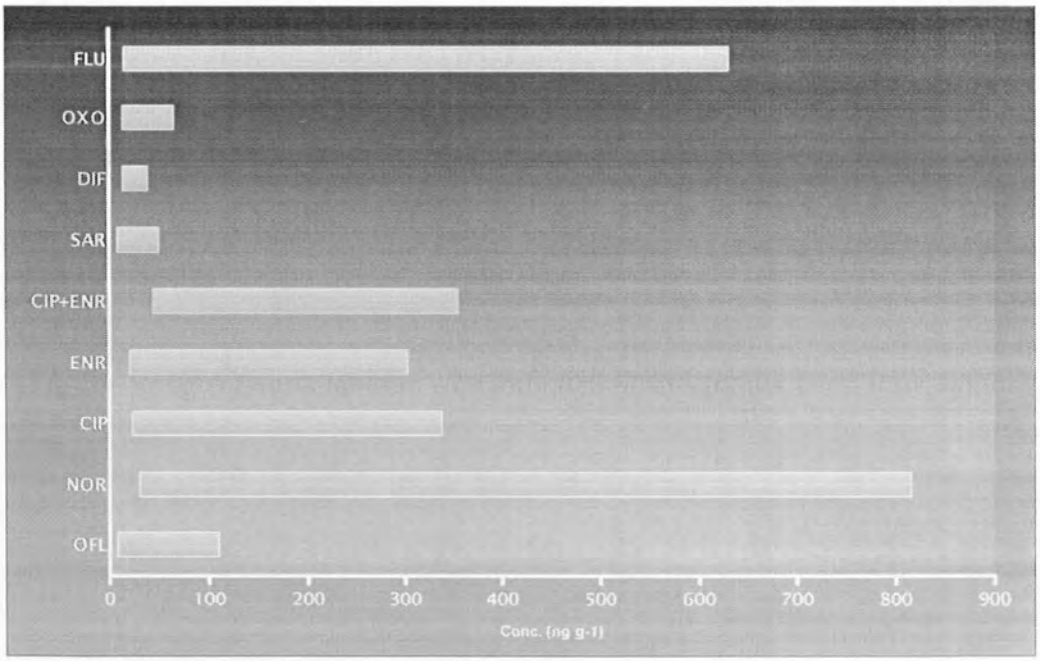


Fig-17: Overall range of quinolones in contaminated meat samples

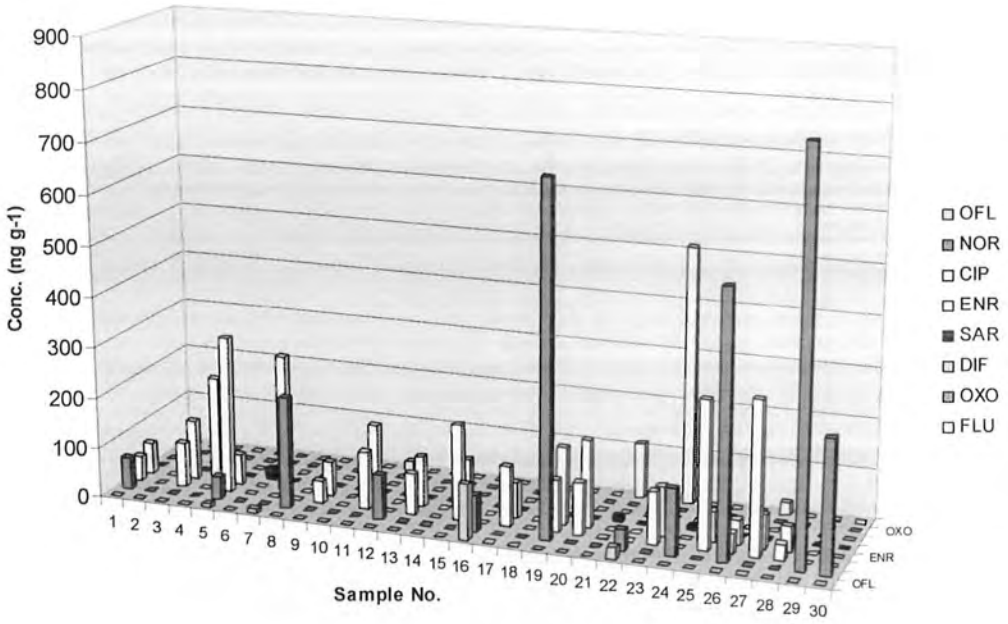


Fig-18: Detailed analysis of samples from Islamabad

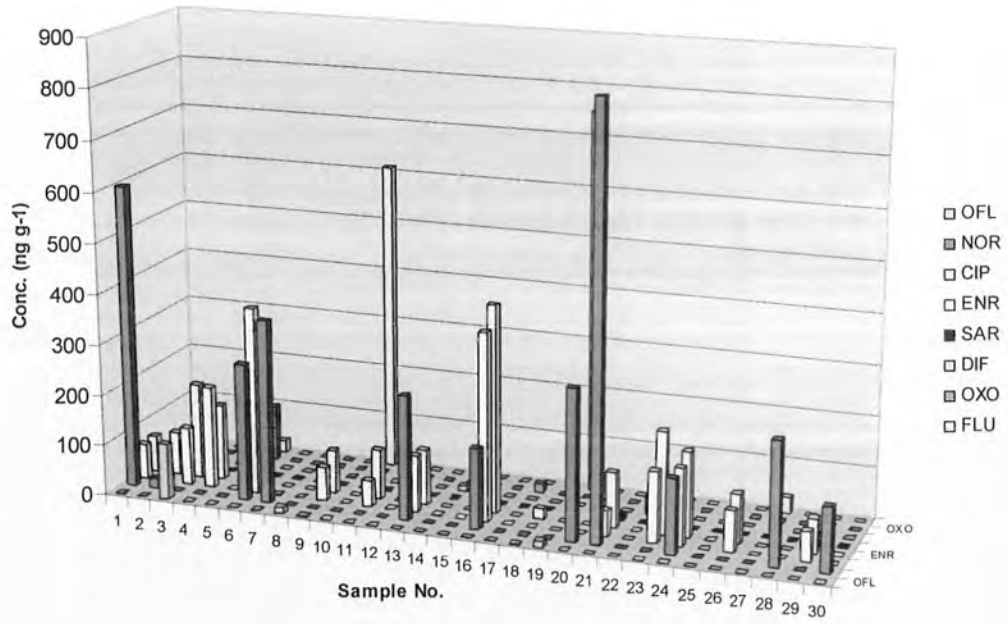


Fig-19: Detailed analysis of samples from Rawalpindi

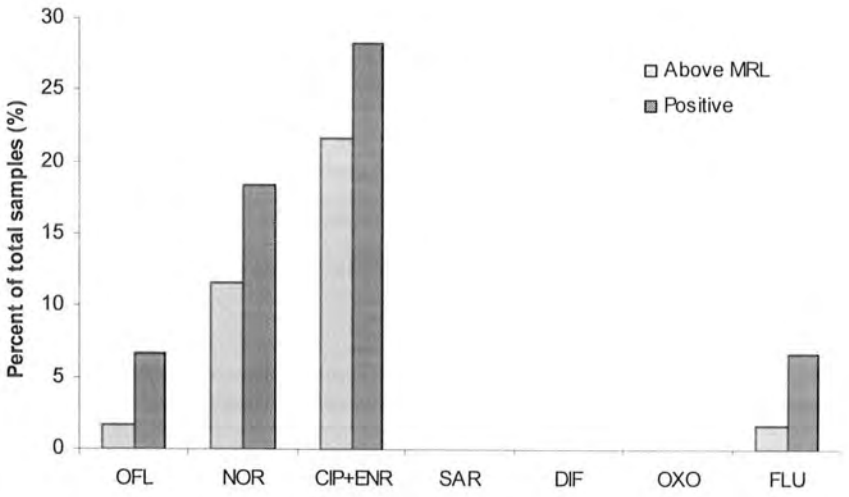


Fig-20: Percent of positive samples and samples above MRL in Islamabad (% of positive samples)

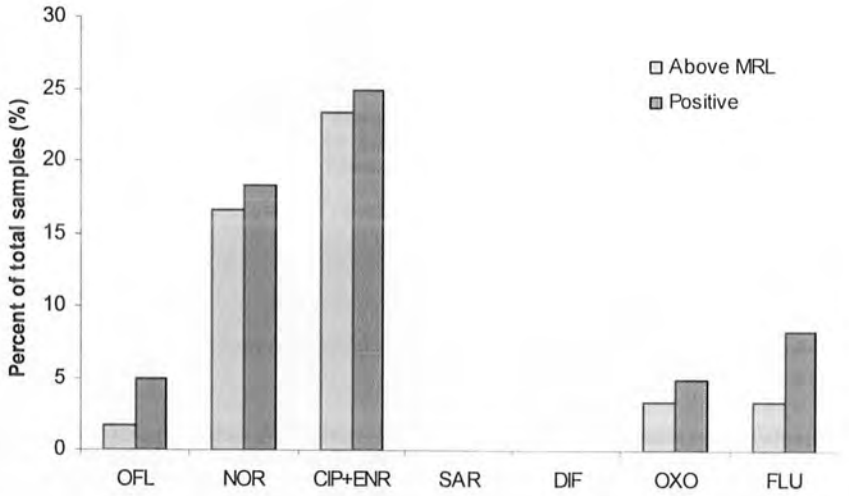


Fig-21: Percent of positive samples and samples above MRL in Rawalpindi (% of positive samples)

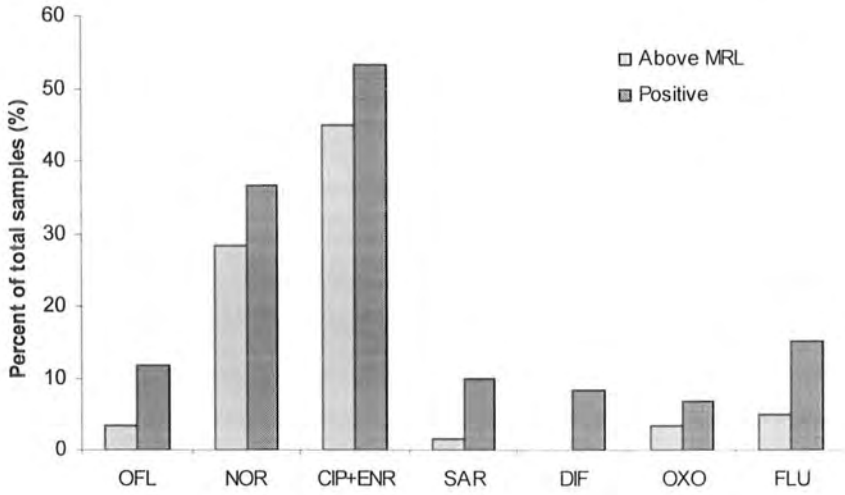


Fig-22: Overall percent of positive samples and samples above MRL in Islamabad/Rawalpindi (% of positive samples)

FTIR analysis:

Tables-34, -35 and Fig-23, -24 compare results oFTIR and HPLC for the meat samples studied for the presence of quinolones residues.

Table-33: Confirmation and comparison of HPLC results with FTIR in meat samples from Islamabad

Sample No.	OFL		NOR		CIP		EFX		SAR		DIF		OXO		FLU	
	HPLC	FTIR	HPLC	FTIR	HPLC	FTIR	HPLC	FTIR	HPLC	FTIR	HPLC	FTIR	HPLC	FTIR	HPLC	FTIR
1.	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-
2.	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
3.	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-
4.	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-
5.	+	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-
6.	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
7.	+	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-
8.	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
9.	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-
10.	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
11.	-	-	-	-	+	+	+	+	-	-	-	-	-	-	+	+
12.	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
13.	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-
14.	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
15.	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-
16.	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
17.	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-
18.	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
19.	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-
20.	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-
21.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
22.	+	+	+	+	-	-	-	-	+	+	-	-	-	-	-	-
23.	-	-	-	-	+	+	+	+	-	-	-	-	-	-	+	+
24.	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-
25.	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-
26.	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-
27.	-	-	-	-	+	+	+	+	-	-	-	-	-	-	+	+
28.	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-
29.	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
30.	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-

Table-34: Confirmation and comparison of HPLC results with FTIR in meat samples from Rawalpindi

Sample No.	OFL		NOR		CIP		EFX		SAR		DIF		OXO		FLU	
	HPLC	FTIR	HPLC	FTIR	HPLC	FTIR	HPLC	FTIR	HPLC	FTIR	HPLC	FTIR	HPLC	FTIR	HPLC	FTIR
1.	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-
2.	-	-	+	-	+	+	+	+	-	-	-	-	-	-	-	-
3.	+	+	-	-	+	+	+	+	-	-	-	-	-	-	-	-
4.	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-
5.	-	-	-	-	-	-	-	-	-	-	+		+		+	+
6.	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-
7.	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
8.	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9.	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-
10.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
11.	-	-	-	-	+	+	+	+	-	-	-	-	+		-	-
12.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13.	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-
14.	-	-	-	-	-	-	-	-	-	-	+		-	-	-	-
15.	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
16.	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-
17.	-	-	-	-	-	-	-	-	-	-	-	-	+		-	-
18.	-	-	-	-	-	-	+		-	-	-	-	-	-	-	-
19.	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
20.	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
21.	-	-	+	+	+	+	+		+	-	-	-	-	-	-	-
22.	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
23.	-	-	-	-	+	+	+		-	-	-	-	-	-		-
24.	-	-	+	+	+	+	+		-	-	-	-	-	-	-	-
25.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
26.	-	-	-	-	+	+	+		-	-	-	-	-	-	-	-
27.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
28.	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
29.	-	-	-	-	+	+	+		-	-	-	-	-	-	-	-
30.	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-

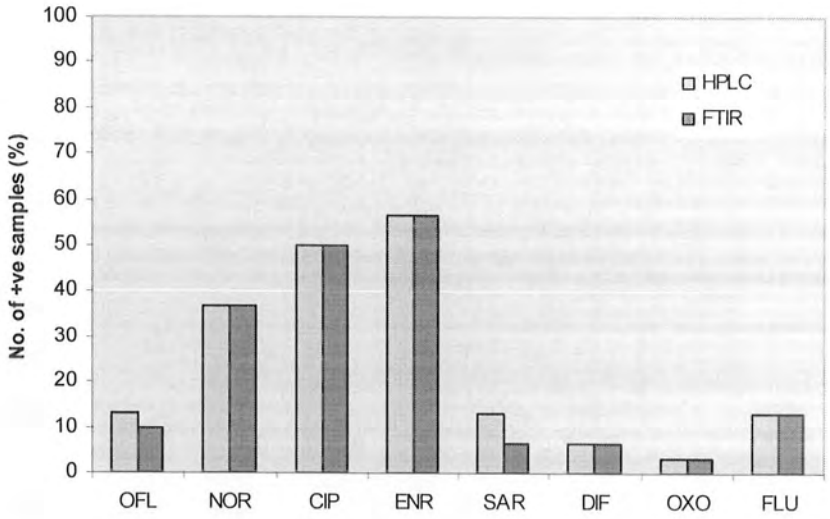


Fig-23: Comparison of HPLC and FTIR results of samples from Islamabad

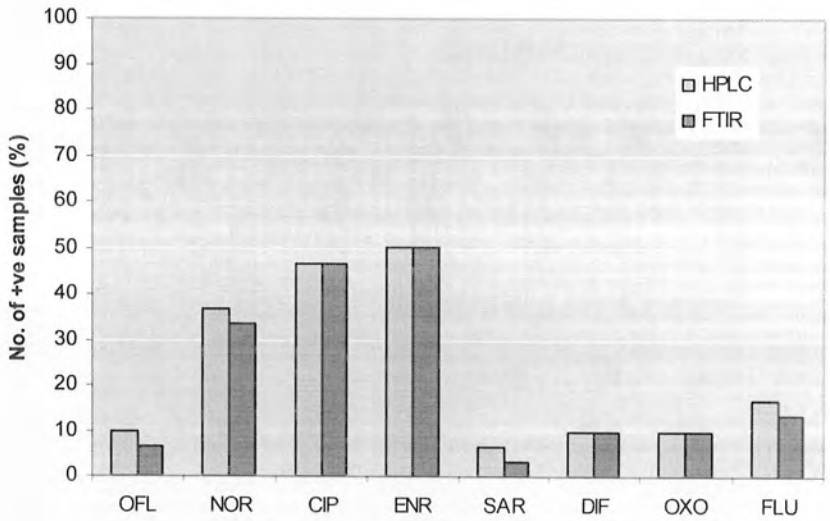


Fig-24: Comparison of HPLC and FTIR results of samples from Rawalpindi

Discussion:

The current study was conducted under multiple objectives. The primary aim was to optimize and validate a method for the reliable extraction and detection of quinolones from the poultry meat. Once a rugged method is achieved, the secondary purpose was to apply this to the real samples and conducting a survey at random on the assessment of violation of quinolones contamination of commercially available broiler poultry meat sold in local markets. This study will, therefore, also help in consumer awareness to the antibiotic residues threat and at the same time will be a guideline and base study for the legislative government bodies to ponder over this aspect of consumer health and safety for the formulation and implementation of more straightforward and stringent actions. Also this will have a solid impact on the export of meat and meat by-products to other countries where strict measures prevail regarding antibiotic residues.

For the detection of quinolone residues various procedures have been employed by different workers like ELISA (Holtzapple *et al.*, 1997), HPLC (Liu *et al.*, 2004; Dong *et al.*, 2005; Bailac *et al.*, 2004; Pecorelli *et al.*, 2003), capillary electrophoresis (Kowalski and Plenis, 2008) and mass spectrometry (Toussaint *et al*, 2005). Dong *et al.* (2005) detected four different quinolones in muscles employing phosphate buffer, with a very good LOQ of 4–20 ng g^{-1}. Bailac *et al.* (2004) found seven different quinolones in tissues, using dichlormethane extraction. Verdon *et al.*, (2005) determined 10 quinolones with trichloracetic acid in chicken by HPLC-FLD. In the current study HPLC system with UV detection was used for the detection of quinolones residues.

The importance and seriousness of residues can be visualized by the number of increasing number of literature in this field in the recent few years. A number of papers have been published addressing this issue. There are papers describing determination of various quinolones in various matrices like egg (Huang *et al.*, 2006; Gigosos *et al.*, 2000; Hassouan *et al.*, 2007; Zeng *et al.*, 2005), chicken muscles (Baillac *et al.*, 2006; Baillac *et al.*, 2004; York and Froc, 2000), eggs and chicken tissues (Schneider and Donogue, 2002; Schneider and Donogue, 2003). For all these methods, HPLC system has been used with various types of detection methods.

Keeping in view the objectives and for convenience, the whole study can be grossly split into two main phases i.e. optimization & validation phase, and application phase.

A. Optimization & Validation Phase

Setting up of Procedure

The first phase was to establish a reliable and reproducible method for the detection of various quinolones antibiotics in poultry meat. The aim was to fully optimize and validate the various procedures to be used. Chromatographic procedures can vary from laboratory to laboratory; therefore, the following study was conducted at National Veterinary Laboratories, Islamabad, which has the mandate to test for the drug residues, to setup a method according to the conditions here.

Preliminary Conditions

Before starting with the main procedures it was necessary to know and setup the basic conditions by using the reagents and facilities available in the laboratory. The later conditions were setup according to the information obtained from these preliminary experiments.

UV/Vis scans of Standards

All the antibiotics (OFL, NOR, CIP, EFX, SAR, DIF, OXO and FLU) were initially screened by the UV/Vis spectrophotometer to work out their absorbance pattern to help set up the chromatographic conditions subsequently. The screening was, therefore, performed in a wide range (200-500 nm) and the maximum absorbance (A_{max}) was recorded for each of the antibiotic. Also the absorbance of the blank (mobile phase) was also determined to make sure that both the analyte and the mobile phase have different absorption pattern and hence do not interfere mutually. The individual scans of the various quinolone antibiotics along with that of mobile phase are shown below. It can be seen that the analytes are absorbing maximum in the range about 250-280 nm while the mobile phase is showing no absorbance in this region and

gives very low absorbance in the low UV area but the working area for all of the analytes is safe. Therefore, these wavelengths were selected for the quinolones with confidence. Except OXO and FLU which are quinolones and have A_{max} around 250 nm, all others antibiotics which are actually fluoroquinolones (having a fluorine atom attached to the central ring system) have their A_{max} around 280 nm and hence a shift in their absorption maxima. For this reason two different wavelengths were selected for HPLC-UV analysis; 280 nm for NOR, CIP, EFX, DAN, SAR, DIF, and 252 nm for OXO, FLU.

Chromatographic conditions

For reversed phase chromatography usually a mobile phase consisting of organic solvents (acetonitrile, methanol) and water with dissolved electrolyte at certain pH are used. The choice of electrolyte depends upon the required pH which should be within the range of ±1 of the pKa value for the maximum buffering capacity.

In the current study mobile phase consisting of methanol, acetonitrile and buffer (citric acid) was used for the best elution and separation of variety of quinolones studied. For the initial gross tuning of peaks separation, different combinations of these ingredients were tried keeping the buffer volume constant with acetonitrile and methanol in reverse increment proportions.

The initial buffer tried was 10 mM citric acid at pH 3.0. Flow rate was 1.2 ml min^{-1} and the UV detection wavelength was 280 nm for all quinolones except OXO and FLU for which 252 nm was set based upon the absorption pattern observed with the UV/Vis spectrophotometer. Separation was aimed at clearly separated peaks with no tailing/fronting, no overlapping and no exceptionally high retention time. Best separation was achieved with the combination (acetonitrile:methanol:buffer 10:12:80). Very nice and sharp peaks were obtained with this combination and pH, however, the only problem was very close retention time for SAR and DIF and these were, therefore, overlapping.

Buffer pH

With the pH 3.0 buffer, all the peaks were well resolved except SAR and DIF. Since the pH of mobile phase is an important variable for good separation of quinolones because of their very identical structure (Si-Jun *et al.*, 2007), therefore, unlike more acidic pH a lesser acidic pH was tried (pH 4.5). At this pH SAR and DIF were separated from each other as clear in the chromatogram below. However, this was not a suitable pH for OFL and NOR as they nearly merged. Therefore, it was not possible to separate all the quinolones under study to separate with isocratic conditions so a gradient was required using two buffers at different pH values (pH 3.5 and 4.5).

Wavelength

Also since the quinoloes have different absorbance values so different detection wavelengths were chosen except oxolinic acid and flumequine for which 252 nm was taken, while 280 nm was selected for others.

Mobile phase

Different levels of acetonitrile and methanol were tried to have a good separation of all the peaks and to reduce the retention time of flumequine as it is strongly retained with the stationary phase (Canada *et al.*, 2007). For the gradient program since the isocratic mobile phase (pH 3.0) worked good until CIP with well resolved peaks so initially the mobile phase was kept acetonitrile:methanol:buffer 10:12:78. After 12 min the buffer was replaced with that of pH 4.5. Also the proportion of organic components was necessary to increase, especially acetonitrile, to attain early elution.

Quinolones consist of polar compounds but vary in polarity. More polar quinolones OFL, NOR and CIP can be easily separated under isocratic conditions; however, for less polar quinolones different conditions are required. Therefore, for the successful separation of all the quinolones at the same time a gradient program is required. Moreover, FLU is highly retained compound because unlike other quinolones it has only one functional group and shows strong association with the nonpolar stationary phase (Baila *et al.*, 2004; Canada *et al.*, 2007. For this reason higher proportion of

acetonitrile and a greater flow rate of mobile phase is required. Moreover, to further get all the peaks early a flow rate of 1.2 ml min^{-1} was used.

Samples preparation

Different extraction strategies have been adopted by different laboratories using different chemicals for maximum recovery (Hernández-Arteseros *et al.*, 2002; Juan-Garcia *et al.*, 2006; Sun *et al.*, 2007; Posyniak *et al.*, 1999; Posyniak *et al.*, 2001); Zhao *et al.*, 2007a; Zhao *et al.*, 2007b). The aim is always to have maximum recovery of analyte of interest with least interfering matrix left over. To get rid of the unwanted impurities in sample cleanup procedure using solid phase extraction system was used. The method-II clearly yielded better in all the cases and therefore this method was chosen and further validated.

Method validation

- Stability studies
- Precision
- Accuracy
- Linearity
- Range
- Recovery
- Limit of Detection (LOD)
- Limit of Quantitation (LOQ)

Stability studies

Monitoring of stability of the analytes in samples is crucial as it determines the ultimate fate of the results. A sample is usually carried to laboratory and stored frozen, so both the factors i.e. sample matrix and storage conditions which can affect the analyte should be validated before proceeding towards the sample analysis so that any alteration introduced by these factors may be ruled out (2002/657/EC, 2002; www.fda.gov).

Freeze-and-thaw stability

To access the stability of the analytes after freezing thawing, three blank meat samples were spiked at 100 ng g^{-1}. Then these samples were subjected to freeze-thaw cycle (frozen for 24h and then thawed) as all the samples were passed through these phases. It was observed that the freezing-thawing had no significant effect on recovery of quinolones.

Freezing period

The effect of freezing duration was studied by spiking blank meat samples at 100 ng g^{-1} in triplicate and then freezing for three weeks as all the samples were collected and stored within this period. Samples were defrosted and analyzed after one day and then after three weeks later. As compared with the expected recovery, the concentrations of the quinolones extracted were not significantly different. This showed that freezing of meat samples for short periods (three weeks here) does not affect these antibiotics. Longer period than this was not studied and was also not required as none of the samples was stored beyond this time limit.

Three extracts from blank meat samples were spiked with a 100 ng g^{-1} concentration of quinolones. These samples were analysed immediately (t=0) and left at room temperature as such until 24h at which times these were again analyzed. The difference between the two time points was calculated for each of the quinolone. This was essential to monitor any effect on the analytes over the period of time they have to stay in the sample vials of the HPLC auto sampler waiting for their turn. The results indicated that there was no significant effect on the conc. of the quinolones studied during the 24h period. The effect of room temperature beyond this time was not studied.

Recovery

A good recovery of all the analytes under study was achieved with the extraction and cleanup procedures used. Three different spike levels were used i.e. 50, 100 and 150 ng g^{-1}.

Precision and accuracy

The precision of an analytical method is the degree of agreement among individual test results obtained when the method is applied to multiple sampling of a homogenous sample. Precision is a measure of the reproducibility of the *whole* analytical method (including sampling, sample preparation and analysis) under normal operating circumstances. Precision is determined by using the method to assay a sample for a sufficient number of times to obtain statistically valid results (i.e. between 6-10). The precision is then expressed as the relative standard deviation:

$$\%RSD = (\text{Standard Deviation} / \text{Mean}) \times 100$$

Accuracy is a measure of the closeness of test results obtained by a method to the true value. (6) Accuracy indicates the deviation between the mean value found and the true value. It is determined by applying the method to samples to which known amounts of analyte have been added. These should be analysed against standard and blank solutions to ensure that no interference exists. The accuracy is then calculated from the test results as a percentage of the analyte recovered by the assay. Accuracy and precision are not the same. A method can have good precision and yet not be accurate (www.standardbase.com).

Linearity and Range

Linearity and range determine the capability of the method to give results within a specified range. This was determined by preparing different concentrations of quinolones standard antibiotics and subsequently running them in HPLC under the conditions worked out for analysis. The peak areas were collected and a graph was

plotted between the peak areas and the concentration corresponding to it. A very good association between these two was observed in all the cases. The correlation coefficient which actually determines the health of the calibration curve was between 0.9989 and 0.9997, slope ranged between 162.6 and 392.5 while the intercept was -685.7 to 471.9.

LOD and LOQ

Limit of detection is the minimum quantity of an analyte that can be detected with a particular instrument but it is not necessary that the same can be quantitated. For more accuracy LOD is usually taken higher than the signals originating from a blank sample (Bailac *et al.*, 2006). There are various ways for calculating LOD based on:

Visual Evaluation

This is usually performed for procedures where no instruments are used.

Signal-to-Noise

This usually used for procedures using analytical instruments like HPLC. There is a baseline and noise of this baseline is important in determining signal-to-noise ratio which is ultimately used for the calculation of LOD and LOQ. The signal-to-noise ratio (5) is determined by:

$$s = H/h$$

Where,

H = peak height

h = the biggest noise fluctuation from the blank baseline

Usually a signal-to-noise ratio of 3:1 or 2:1 are selected to differentiate a signal from analyte from that of baseline. LOQ is in general 10 times of noise signal which is higher enough to be sure that the signal is actually from analyte and not due to noise in detector.

Standard Deviation of the Response and the Slope

In this method LOD is determined by the relation:

$$DL = 3.3\ \sigma / S$$

Where,

σ = the standard deviation of the response

S = the slope of the calibration curve

A good LOD and LOQ were achieved although compared to other these were higher. However these were still well below the MRLs.

B. Application to Real Samples

HPLC analysis:

Once the whole method for the detection of selected quinolones was optimized and validated, it was applied to the real samples (poultry meat) randomly collected from various markets of Rawalpindi/Islamabad. A total of 60 samples were tested for the residual contamination of various quinolone antibiotics frequently administered to the commercial broilers. Most of the samples were found contaminated with some type of quinolone antibiotic. The samples were grossly divided into two different categories; those from Rawalpindi and those from Islamabad for the sake of simplicity.

Overall, out of 60 samples tested, 11.7, 36.7, 48.4, 48.5, 10, 8.4, 6.7 and 15% samples were found to be contaminated with OFL, NOR, CIP, ENR, SAR, DIF, OXO and FLU respectively with 29.1, 77.3, 84.9, 50, 0, 33.3 and 32.5% of these samples being above MRL set by EU/FAO.

FTIR analysis:

Fourier transform infrared spectrophotometer (FTIR) identification of the eluted peaks from the HPLC was performed as a secondary check and confirmation. A good

correlation was seen between the HPLC and FTIR results but not 100% as some of the samples positive with HPLC were negative by FTIR. This may be possible because of the co eluting peaks or substances having similar retention time. FTIR was able to identify such false peaks. Overall, seven samples were identified with this problem. Therefore, combined with FTIR, HPLC can give more accurate results.

For secondary confirmation all the samples tested with HPLC were also analyzed by FTIR. A good correlation was found between the HPLC and FTIR results, however, 7 of 60 (11.7%) samples detected with HPLC were found negative with FTIR.

The results of the study show widespread misuse of quinolone antibiotics in broiler poultry farming and is not only a concern for the consumer health but also a main hurdle in export of poultry products and by-products to other countries as presence of such residues beyond recommended levels is a serious issue and such food is banned for human consumption. It is now the responsibility of the government and authorities in force to make sure such violations do not happen. This will have tremendous effect on health and economy.

2002/657/EC. Implementing Council Directive 96/23/EC Concerning the Performance of Analytical Methods and the Interpretation of Results [Internet] 2002 August 12 [cited 2011 Jan]. Official Journal of the European Communities. Report No. 2002/657/EC. Available from:
www.eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2002:221:0008:0036:EN:PDF

Ambrose P.G. and R.C. Owens, Jr. Clinical usefulness of Quinolones. Seminars in Respiratory and Critical Care Medicine (Medscape) [Internet]. 2000 [cited Jan 2011]. Available from: www.medscape.com/viewarticle/410872.

Bailac S., D. Barron and J. Barbosa (2006). New extraction procedure to improve the determination of quinolones in poultry muscle by liquid chromatography with ultraviolet and massspectrometric detection. Analytica Chimica Acta 580 163–169.

Bailac, S., O. Ballesteros, E. Jiménez-Lozano, D. Barrón, V. Sanz-Nebot, A. Navalón, J. Vílchez and J. Barbosa (2004). Determination of quinolones in chicken tissues by liquid chromatography with ultraviolet absorbance detection. *J. Chromatogr. A.* 1029(1-2): 145–151.

Canada, F.C., A.E. Mansilla and A.M. de la Pena (2007). Separation of fifteen quinolones by high performance liquid chromatography: Application to pharmaceuticals and ofloxacin determination in urine. J. Sep. Sci. 30, 1242-1249.

Childs, S.J. (2000). Safety of the Fluoroquinolone Antibiotics: Focus on Molecular Structure. Infect. Urol. (USA: FQresearch) 13(1): 3–10.

De Sarro, A. and G. De Sarro (2001). Adverse reactions to fluoroquinolones. an overview on mechanistic aspects. Curr. Med. Chem. 8(4): 371–84.

Dong, L.L., Y.H. Liu, X. Wang, F. Zhong, L Peng, X.Y. Yue and L. Gao (2005).

Simultaneous determination of four fluoroquinolone residues in edible chicken tissues by reversed-phase high performance liquid chromatography. *Chinese. J. Chromatogr.*, 5(23): 285–288.

Gigosos, P.G., P.R. Revesado, O. Cadahia, C.A. Fente, B.I. Vazquez, C.M. Franco and A. Cepeda (2000). Use of the diphasic dialysis as a new extraction procedure in the determination of enrofloxacin and ciprofloxacin in egg. J. Chromatogr. A. 871(1-2): 31-36.

Hassouan, M.K., O. Ballesteros, J. Taoufiki, J.L. Vilchez, M. Cabrera-Aguilera and A. Navalon, J. (2007). Multiresidue determination of quinolone antibacterials in eggs of laying hens by liquid chromatography with fluorescence detection. Chromatogr. B. 852(1-2): 625-630.

Hernández-Arteseros, J.A., J. Barbosa., R. Campañó and M.D. Prat (2002). Analysis of quinolone residues in edible animal products. J. Chromatogr. A. 945, 1-24.

Holtzapple, C.K., S.A. Buckley and L.H. Stanker (1997). Production and characterization of monoclonal antibodies against sarafloxacin and cross-reactivity studies of related fluoroquinolones J. Agric. Food Chem. 45, 1984– 1990.

Hooper, D.C. (2001). Emerging mechanisms of fluoroquinolone resistance. Emerg. Infect. Dis. 7(2): 337–41.

Horii T., A. Monji, K. Uemura and O. Nagura (2006). Rapid detection of fluoroquinolone resistance by isothermal chimeric primer-initiated amplification of nucleic acids from clinical isolates of Neisseria gonorrhoeae. *J. Microbiol. Methods.* 65(3): 557–561.

Huang, J.F., B. Lin, Q.W. Yu and Y.Q. Feng (2006). Determination of fluoroquinolones in eggs using in-tube solid-phase microextraction coupled to high-performance liquid chromatography. Anal. Bioanal. Chem. 384(5): 1228-1235.

Ivanov D.V. and S.V. Budanov (2006). Ciprofloxacin and antibacterial therapy of respiratory tract infections (in Russian). Antibiot. Khimioter. 51 (5): 29–37.

Jacobs M.R. (2005). Antimicrobial Agents and Resistance--Fifth International Symposium. IDrugs. 8(7):542-546.

Javed, M. (1988). Residues of sulfa chloropyrazin in poultry products. M.Sc. Thesis (Veterinary Microbiology), University of Agriculture, Faisalabad, Pakistan.

Juan-Garcia, A., G. Font and Y. Pico (2006). Determination of quinolone residues in chicken and fish by capillary electrophoresis-mass spectrometry. Electrophoresis. 27, 2240-2249.

Kowalski, P. and A. Plenis (2008). Simultaneous determination of six quinolone antibiotics in poultry and porcine samples by capillary electrophoresis. Bull. Vet. Inst. Pulawy. 52, 81-85.

Lautenbach E., L.A. Larosa, N. Kasbekar, H.P. Peng, R.J. Maniglia, N.O. Fishman (2003). Fluoroquinolone utilization in the emergency departments of academic medical centers: prevalence of, and risk factors for, inappropriate use. Arch. Intern. Med. 163 (5): 601–605.

Leverkusen A.G. Mechanism of Action. [Internet]. Bayer. 1990. [cited Jan 2011]. Available from:
www.animalhealth.bayerhealthcare.com/fileadmin/media/baytril/pdf_food/kap3.pdf.

Linder J.A., E.S. Huang, M.A. Steinman, R. Gonzales and R.S. Stafford (2005). Fluoroquinolone prescribing in the United States: 1995 to 2002. Am. J. Med. 118(3): 259–268.

Liu, H. and Mulholland, S.G. (2005). Appropriate antibiotic treatment of genitourinary infections in hospitalized patients. Am. J. Med. 118 Suppl 7A: 14S–20S.

Liu, Y., M. Xie, L. Ding, J. Shan, Q. Yang and S. Liu (2004). Simultaneous determination of four fluoroquinolones in eggs by high performance liquid chromatography. Fenxi. Huaxue. 32(3): 352–355.

MacDougall C., B.J. Guglielmo, J. Maselli and R. Gonzales (2005). Antimicrobial drug prescribing for pneumonia in ambulatory care. Emerging Infect. Dis. 11(3): 380-384.

Morita Y., K. Kodama, S. Shiota, T. Mine, A. Kataoka, T. Mizushima and T. Tsuchiya (1998). NorM, a putative multidrug efflux protein, of Vibrio parahaemolyticus and its homolog in Escherichia coli. Antimicrob. Agents Chemother. 42 (7): 1778–82.

Naeem, M., K. Khan and S. Rafiq (2006). Determinatio of residues of quinolones in poultry products by high performance liquid chromatography. J. App. Sc. 6(2): 373-379.

Nelson J.M., T.M. Chiller, J.H. Powers and F.J. Angulo (2007). Fluoroquinolone-resistant Campylobacter species and the withdrawal of fluoroquinolones from use in poultry: a public health success story. Clin. Infect. Dis. 44(7): 977–980.

Neuhauser M.M., R.A. Weinstein, R. Rydman, L.H. Danziger, G. Karam and J.P. Quinn (2003). Antibiotic resistance among gram-negative bacilli in US intensive care units: implications for fluoroquinolone use. J. Am. Med. Ass. 289 (7): 885–888.

Niwa H., T. Chuma, K. Okamoto and K. Itoh (2003). Simultaneous detection of mutations associated with resistance to macrolides and quinolones in Campylobacter jejuni and C. coli using a PCR-line probe assay. *J. Antimicrob. Agents*. 22(4): 374–379.

Norris, S and G.L. Mandell (1988). The quinolones: history and overview. The quinolones: history and overview. San Diego: Academic Press Inc. pp. 1–22.

Oliphant C.M. and G.M. Green (2002). Quinolones: A Comprehensive Review. Am Fam Physician. 65(3): 455-465.

Owens R.C. and P.G. Ambrose (2005). Antimicrobial safety: focus on fluoroquinolones. Clin. Infect. Dis. 41(2): S144–57.

Pecorelli, I., R. Galarini, R. Bibi, A. Floridi, E. Casciarri and, A. Floridi (2003). Simultaneous determination of 13 quinolones from feeds using accelerated solvent extraction and liquid chromatography. *Anal. Chim. Acta*, 2003, 483: 81–89.

Posyniak, A., J. Żmudzki. and S. Semeniuk (2001). Effects of the matrix and sample preparation on the determination of fluoroquinolone residue in animal tissues. *J. Chromatogr. A.* 914, 89-94.

Posyniak, A., J. Żmudzki., S. Semeniuk., S. Niedzielska and R. Eblis (1999). Determination of fluoroquinolone residues in animal tissues by liquid chromatography. Biomed. Chromatogr. 13, 279-285.

Robicsek A., G.A. Jacoby and D.C. Hooper (2006). The worldwide emergence of plasmid-mediated quinolone resistance. Lancet Infect. Dis. 6 (10): 629–640.

Samanidou, V., E. Evaggelopoulou, M. Trotzmuller, X. Guo and E. Lankmayr (2008). Multi-residue determination of seven quinolones antibiotics in gilthead seabream using liquid chromatography–tandem mass spectrometry. J. Chromatog. A, 1203(2): 115–123.

Schneider, M.J. and D.J. Donogue (2002). Multiresidue analysis of fluoroquinolone antibiotics in chicken tissue using liquid chromatography-fluorescence-multiple mass spectrometry. J. Chromatogr. B. 780(1): 83-92.

Schneider, M.J. and D.J. Donogue (2003). Multiresidue determination of fluoroquinolone antibiotics in eggs using liquid chromatography-fluorescence-mass spectrometry. Anal. Chim. Acta. 483(1-2): 39-49.

Si-Jun, Z., L. Cun, J. Hai-Yang, L. Bing-Yu and S. Jian-Zhong (2007). Simultaneous Determination of 7 Quinolones Residues in Animal Muscle Tissues by High Performance Liquid Chromatography. Chin. J. Anal. Chem. 35(6): 786–790.

Sun, H., P. He., Y. Lv and S. Liang (2007). Effective separation and simultaneous determination of seven fluoroquinolones by capillary electrophoresis with diode-array detector. J. Chromatogr. B. 852, 145-151.

Toussaint, B., M. Chedin, U. Vincent, G. Bordin and A. Rodriguez (2005). Determination of (fluoro)quinolone antibiotic residues in pig kidney using liquid chromatography-tandem mass spectrometry. Part II. Intercomparison exercise. *J Chromatogr. A*, 1088(1-2): 40–48.

Verdon, E., P. Couedor, B. Roudaut and P. Sandérs (2005). Multiresidue method for simultaneous determination of ten quinolone antibacterial residues in multimatrix/multispecies animal tissues by liquid chromatography with fluorescence detection: Single laboratory validation study *J. AOAC. Int.*, 88(4): 1179–1192.

WHO/EMC/ZDI/98.10. Use of Quinolones in Food Animals and Potential Impact on Human Health. Report of a WHO Meeting, Geneva, Switzerland 2-5 June 1998. Report No. WHO/EMC/ZDI/98.10.

www.fda.gov. FDA Announces Final Decision About Veterinary Medicine. FDA News. [Internet] 2005 July 28 [cited 2011 Jan]. Available from: www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/2005/ucm108467.htm.

www.merckmanuals.com. Fluoroquinolones [Internet]. 2011 [cited Jan 2011]. Available from: www.merckmanuals.com/professional/infectious_diseases/bacteria_and_antibacterial_drugs/fluoroquinolones.html.

www.mombu.com. The Fluoroquinolone Drugs are the most toxic and dangerousantibiotic in clinical practice today [Internet] 2011. [cited 2011 Jan]. Available from: www.mombu.com/medicine/medicine/t-the-fluoroquinolone-drugs-are-the-most-toxic-and-dangerousantibiotic-in-clinical-practice-today-tuberculosis-psychosis-renal-epidermal-malaise-4480258-last.html.

www.noah.co.uk. Maximum Residue Limits (MRLs) and the Safety of Food from Animals [Internet]. 2011 [cited Jan 2011]. Available from: www.noah.co.uk/issues/briefingdoc/09-mrls.htm.

www.standardbase.com. A Guide to Validation in HPLC [Internet] 2011 [cited 2011 Jan]. Available from: www.standardbase.com/tech/HPLC%20validation%20PE.pdf.

York, J.C. and P. Froc (2000). Quantitation of nine quinolones in chicken tissues by high-performance liquid chromatography with fluorescence detection. J. Chromatogr. A. 882(1-2): 63-77.

Zeng, Z., A. Dong, G. Yang, Z. Chen and X. Huang (2005). Simultaneous Determination of 7 Quinolones Residues in Animal Muscle Tissues by High Performance Liquid Chromatography. J. Chromatogr. B. 821(2): 202-209.

Zhao, S., H. Jiang, X. Li., T. Mi., C. Li. And J. Shen (2007a). Simultaneous determination of trace levels of 10 quinolones in swine, chicken, and shrimp muscle tissues using HLPC with programmable fluorescence detection. J. Agric. Food Chem. 55, 3829-3834.

Zhao, S.J., C. Li., H.Y. Jiang., B.Y. Li and J.Z. Shen (2007b). Simultaneous determination of 7 quinolones residues in animal muscle tissues by high-performance liquid chromatography. Chin. J. Anal. Chem. 35, 786-790.