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Standardization of high performance thin layer chromatographic (HPTLC) technique for detecting antibiotic residues in carp (*Catla catla*) and Gold fish (*Carassius auratus*)

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Abstract
Aim: The aim of this study was to standardize a High Performance Thin Layer Chromatographic Technique for detection and quantification of the antibiotic Oxytetracycline in Carp and Enrofloxacin and its metabolite Ciprofloxacin in Goldfish.
Methodology: Carp and Goldfish samples were collected from the fish market Chennai. Carp samples were extracted for the antibiotic Oxytetracycline, and Goldfish samples were extracted for the antibiotic Enrofloxacin and Ciprofloxacin and detected using a CAMAG HPTLC Scanner-3.
Results: The limit of detection and limit of quantification were ascertained for the antibiotics by High Performance Thin Layer Chromatography. The recovery percentage recorded is above 85% for the antibiotics. Linearity, standard peak and Rf value for the antibiotics were also obtained.
Interpretation: This study explains that High Performance Thin Layer Chromatography has a unique advantage of high throughput, economical and a simple analytical tool that can be used for the analysis of antibiotic residues.

Keywords: Antibiotic residues, Carp, Goldfish, HPTLC

1. Introduction
Aquaculture is one of the most important food producing sectors in the world and it accounts for about 80.0 million tonnes of food fish in 2016 (FAO, 2018) [11]. But, due to the intensive culture, there is a sporadic disease outbreak in fish. Hence, this warrants the use of antibiotics in aquaculture as both therapeutic and prophylactic agent though they have a negative impact on fish and the environment (Alderman and Hastings, 1998; Cabello, 2006) [1, 3].
In aquaculture sector, the widespread use of antibiotics for treating bacterial infections has lead to increase of antibiotic resistance in *Aeromonas hydrophila*, *A. salmonicida*, *Edwardsiella tarda*, *E. icttaluri*, *Vibrio anguillarum*, *V. salmonicida*, *Pasteurella piscida* and *Yersinia ruckeri* (De Paola *et al.*, 1995) [7]. The presence of antibiotic residues in aquaculture products leads to the development of bacterial resistance and could cause toxicity to consumers that can lead to morbidity and death. Chloramphenicol residues, for example, in very low concentrations may activate aplastic anemia. Other toxic effects include immunopathological effects and carcinogenicity by oxytetracycline (EU Commission, 2001) [9].
The pathogenic bacterial resistance to antibacterial is an emerging problem in human and veterinary medicine, antibacterial use in aquaculture is an area of increasing concern over health risks (Kemper, 2008; Mulcahy, 2011) [16, 25]. The above use of antibacterials in fish aquaculture is directly proportional to the increase of resistance in bacteria that can infect both humans and animals (Burridge *et al.*, 2010; Kummerer, 2008; Defoirdt *et al.*, 2011) [2, 20, 6]. Aquaculture is thought to provoke the spread and balance of antibacterial resistance in the environment (Sapkota *et al.*, 2008) [30]. Antibacterial resistant bacteria are more likely to be present in the water and sediment which is associated with aquaculture (Kostich and Lazorchak, 2008) [18].
Enrofloxacin and its metabolite ciprofloxacin is a synthetic antimicrobial agent from the fluoroquinolone family which is extensively used as veterinary medicine. Quinolones have a broad spectrum activity and it especially works against gram-negative bacteria, such as *Aeromonas salmonicida, Renibacterium salmoninarum, Vibrio anguillarum* and intracellular

organisms such as rickettsial, Chlamydia and mycoplasma. Fluoroquinolones are applicable for treating large number of infections in livestock and fish farm (Hanwen Sun *et al.*, 2011) [12]. It is one of the most often recommended antibiotics in fisheries (Samanidou and Evaggelopoulou, 2007) [31], however their use in aquaculture pose a risk to fish products and consumers, because of future risk of residues in the food. Tissue disposition of enrofloxacin and ciprofloxacin, in fish species rely upon many factors such as ambient temperature, water and salinity, properties of drug molecule and lipid content (Haritova and Fink-Gremmels, 2010; Liang *et al.*, 2012) [13, 21]. These factors have a powerful impact on the depletion of residues and withdrawal time from muscles and are mandatory for the presence of unacceptable levels of drugs (Ralica Kyuchukova *et al.*, 2016) [27]. These powerful antibiotics play a major role in the transmission of resistant microorganisms from animals to humans through food chain (Hanwen Sun *et al.*, 2011) [12]. Now a day's enrofloxacin is widely used in ornamental fish for treating bacterial infections (Stoffregen *et al.*, 1997) [32].

Oxytetracycline, a broad spectrum antibiotic invented in 1940s, works against a large variety of bacteria (Chopra and Roberts, 2001) [4]. This drug is mainly used for the prevention and treatment of bacterial disease in fish such as ulcer, furunculosis, redmouth ulcer, etc., (Lida Rafati *et al.*, 2017) [22]. Indiscriminate use of oxytetracycline in aquaculture could lead to unacceptable deposition of their residues in edible tissues (Perrin-Guyomard *et al.*, 2001) [26]. Residues in aquaculture products can affect the market and export value (Heuer *et al.*, 2009; Sapkota *et al.*, 2008) [15, 30]. Oxytetracycline residues are very stable in marine sediments over a period of months (Toutain *et al.*, 2010) [35]. Oxytetracycline produces immunosuppression in some fish species (Svobodova *et al.*, 2006) [34].

MRLs for antibiotics in fish tissues for Oxytetracycline: 100 µg kg^{-1}(muscle), 300 µg kg^{-1} (liver) and 600 µg kg^{-1} (kidney) and for Enrofloxacin and Ciprofloxacin: 100 µg kg^{-1} (muscle + skin) and 200 µg kg^{-1} in liver and kidney (Commission Regulation (EU), 2009) [5]. The Commission of the European Union has established MRL of 100 µg kg^{-1} for enrofloxacin and ciprofloxacin in fish tissue (muscle + skin) (European Economic Commission, 2002) [10].

HPTLC has a unique advantage of high throughput, economical and a simple analytical tool that can be used for the detection of antibiotic residues. This study was aimed to standardize a High-Performance Thin-Layer Chromatographic method for the quantification of antibiotic residues in fish.

2. Materials and Methods

2.1 Sample collection

Freshly slaughtered Carp and Goldfish samples were collected from the fish market and aquarium respectively in the Chennai region. The collected fish samples were labeled accordingly and kept in the deep freezer until it was further used.

Fig 1: Collection of samples (Gold fish and Liver, Kidney and Muscle from Carp)

2.2 Extraction for oxytetracycline and enrofloxacin/ ciprofloxacin

The purity of the solvents was checked by running the solvents in the blank analysis. Certified Reference Material (CRM) of the antibiotic standard Oxytetracycline, Enrofloxacin, and its metabolite Ciprofloxacin were obtained from M/S Neospark drugs and chemicals private limited with a purity standard of 99.9%. The standards were prepared and labeled in amber colored volumetric flasks and kept in stock. Working standards were suitably diluted for HPTLC analysis. Pre-coated aluminum oxide plates (10×8 cm- M/S, E-Merk (India) Ltd.) were used for spotting three samples against authenticated reference standards.

Oxytetracycline extraction was done as per the method described by Kodimalar *et al.*, (2018) [17] with some minor modifications. The fish samples were dissected and muscle, liver and kidney from fish were collected. 4 g of each sample was weighed using a weighing balance and stored in a sample bottle. All the samples (Liver, kidney and Muscle) were spiked with 1 ml of the oxytetracycline standard in the concentration of about 50 µg kg^{-1} which is below the Maximum Residue Limit for oxytetracycline followed by 10 ml of buffer which was added to each sample and shaken well in an orbital shaker for 30 minutes. The samples were homogenized by using homogenizer. Samples were then centrifuged at 5000 rpm for 15 minutes using Remi Centrifuge. The resultant aqueous supernatant was filtered through Whatman filter paper No.1 and collected separately. The collected aqueous supernatant was subjected to liquid-liquid extraction by adding 32 mL of dichloromethane in a 500 ml separating funnel. The resultant organic extract was passed through sodium sulfate bed and collected in a beaker. It is later concentrated in a hot plate kept under the fume hood. Finally, the dried extract was reconstituted with 200 µl of dichloromethane and subjected to oxytetracycline quantification by High-Performance Thin-Layer Chromatography.

Enrofloxacin and Ciprofloxacin were extracted as per the method described by Kumar (2012) [19]. The fish samples were dissected and muscle was collected. 4 g of the sample was weighed using a weighing balance and stored in a sample bottle. The sample was spiked with 1 ml of enrofloxacin and ciprofloxacin standard in the concentration of about 100 µg kg^{-1} which is below the Maximum Residue Limit of enrofloxacin and ciprofloxacin followed by 16 ml of 5 % aqueous trichloroacetic acid (TCA) which was added to the sample and shaken well in an orbital shaker for 30 minutes. The samples were homogenized by using homogenizer. Samples were then centrifuged at 5000 rpm for 15 minutes using Remi Centrifuge. The resultant aqueous supernatant was filtered through Whatman filter paper No.1 and collected separately. The collected aqueous supernatant was subjected to liquid-liquid extraction by adding 32 mL of dichloromethane in a separating funnel. The organic fraction-I was collected separately in the beaker and left out aqueous fraction was once again extracted with 10 mL of dichloromethane. The organic fraction-II obtained was added to the fraction I. The resultant organic extract was passed through sodium sulfate bed and collected in a beaker and concentrated in a hot plate under the fume hood. Finally, the dried extract was reconstituted with 200 µl of dichloromethane and subjected to enrofloxacin and ciprofloxacin quantification by HPTLC.

2.3 High-performance thin-layer chromatographic technique

HPTLC was done as per the method used by Sureshkumar *et al.*, (2017) [33]. Thin silica gel plate was cut to the size (10×8 cm) and marked with a pencil at the upper edge of the plate for the direction of development. The plate was completely washed with methanol and 5% EDTA for detection of oxytetracycline and kept in a hot plate for drying. The plate used for enrofloxacin and ciprofloxacin detection does not need any pre wash. The samples were spotted (spray-on-technique) using an injector by using Linomat-5 sample applicator. The volume used for spotting was approximately 20 µl. The spotted sample plate was kept in a developing chamber for development which contained the developing solvent. The developing solvent used for oxytetracycline was 13:4:2 ratio of Dichloromethane: Acetonitrile: 5% EDTA and for enrofloxacin and ciprofloxacin it was 2:3:2:3 ratio of Dichloromethane: Methanol: 25% aqueous ammonia: Acetonitrile. The spotted samples were developed up to 80mm from the lower edge of the plate. The developed plates were dried and exposed to hydrochloric acid fumes (for enrofloxacin and ciprofloxacin). The plates were viewed under UV light. The plates were then scanned using CAMAG HPTLC scanner-3 under 366nm wavelength to obtain the required results such as linearity, recovery, and repeatability.

3. Results and Discussion

The study revealed the limit of detection and limit of quantification of HPTLC for the antibiotics. Limit of detection for oxytetracycline was 5 ng/spot, and for enrofloxacin and ciprofloxacin, it was 5 ng/spot. The Limit of quantification for oxytetracycline was 10 ng/spot, and for enrofloxacin and ciprofloxacin, it was 5 ng/spot. It has been calculated that the recovery percentage for all the antibiotics were above 85%. Linearity and repeatability were also obtained. The Rf value for oxytetracycline, enrofloxacin and ciprofloxacin were 0.16, 0.64 and 0.59, respectively.

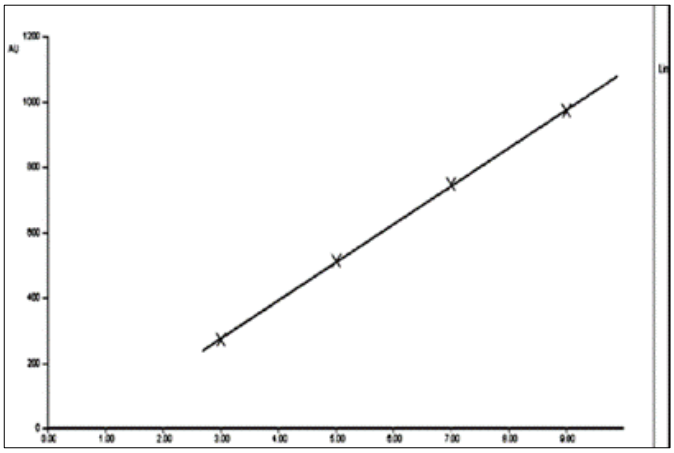

Fig 2: Linearity of oxytetracycline (Same concentration and same volume)

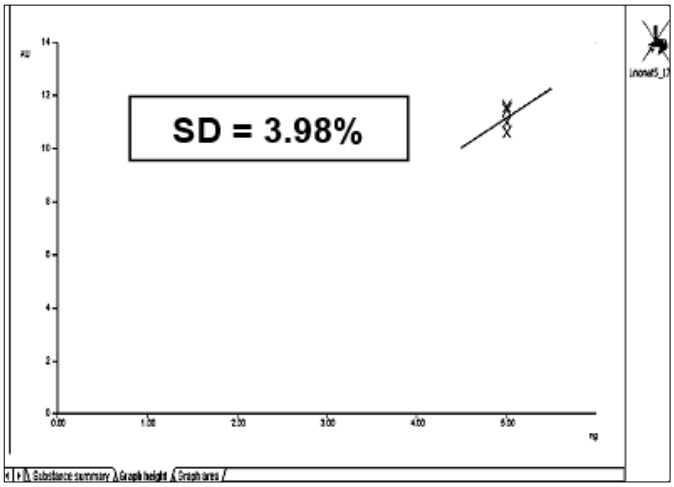

Fig 3: Repeatability of oxytetracycline OTC in spiked sample falls in same area (Used same concentration and different volumes).

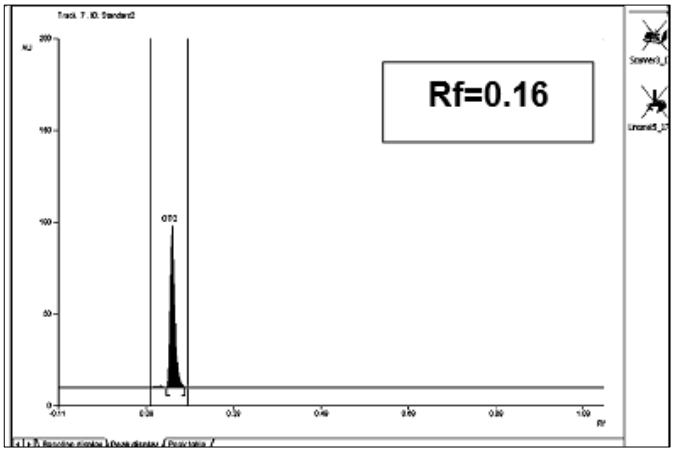

Fig 4: Standard peak of oxytetracycline

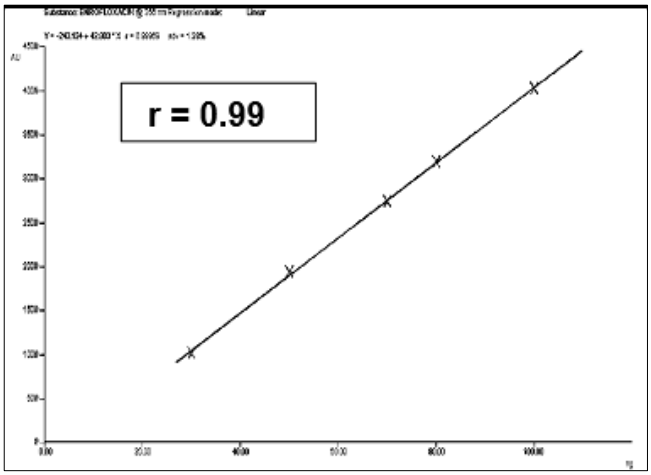

Fig 5: Linearity of Enrofloxacin (Same concentration and same volume)

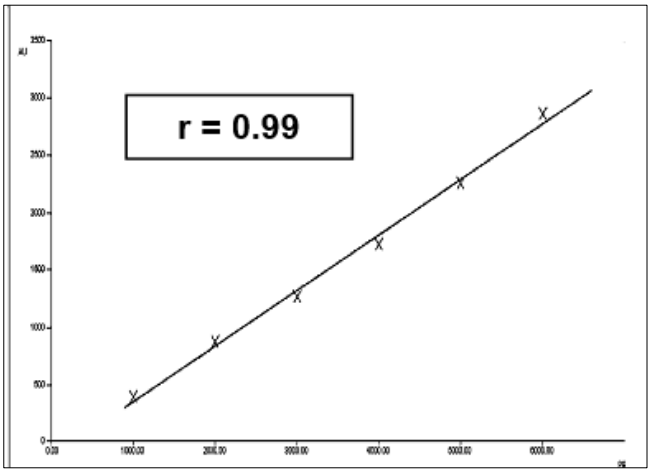

Fig 8: Linearity of Ciprofloxacin (Same concentration and same volume).

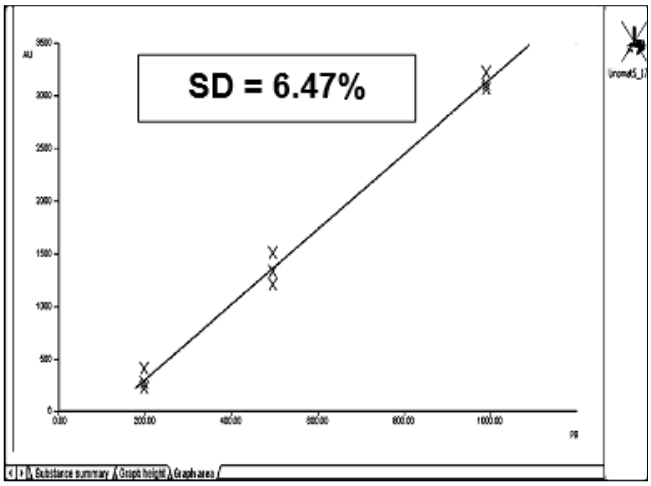

Fig 6: Repeatability of Enrofloxacin Enrofloxacin in spiked sample falls in same area. (Used same concentration and different volumes).

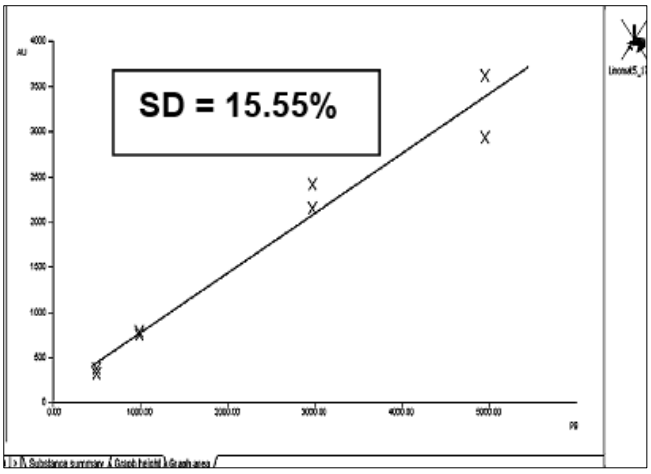

Fig 9: Repeatability of Ciprofloxacin Ciprofloxacin in spiked sample falls in same area. (Used same concentration and different volumes).

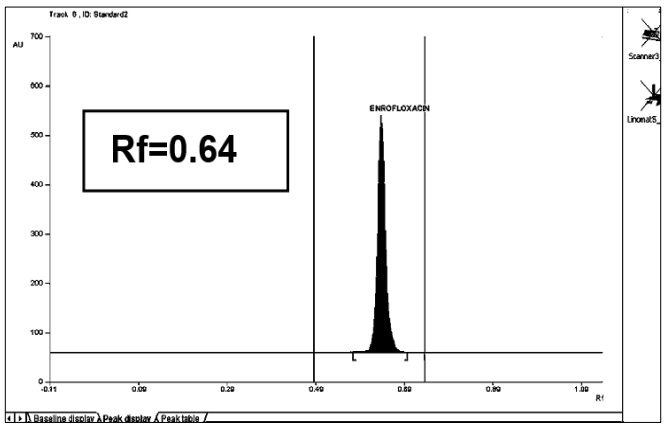

Fig 7: Standard peak of Enrofloxacin

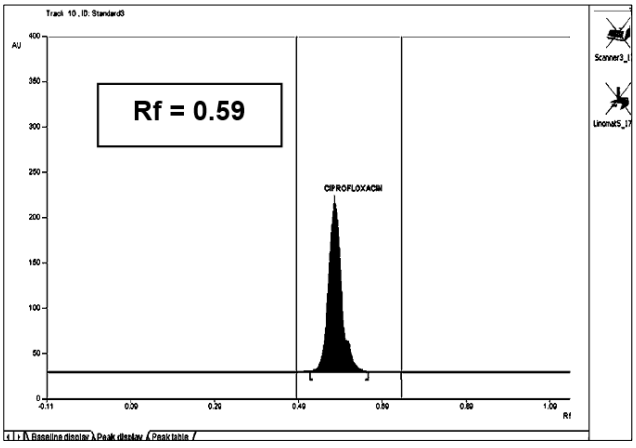

Fig 10: Standard peak of Ciprofloxacin

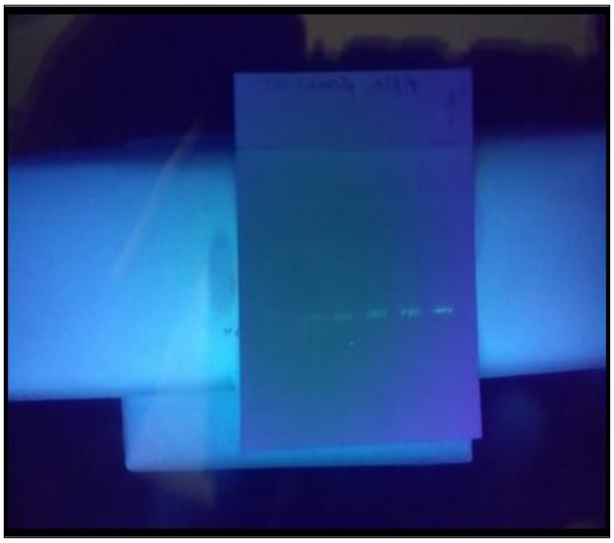

Fig 11: TLC plate showing linearity of oxytetracycline under UV.

Fig 12: TLC plate showing linearity of enrofloxacin under UV

Fig 13: TLC plate showing linearity of ciprofloxacin under UV

An HPTLC method is a robust, rapid, simplest and efficient tool used for the quantitative analysis of compounds. It offers better resolution and lower limit of detection. HPTLC is the most reliable, flexible and cost-efficient separation technique that is mainly suitable for the quantitative analysis of herbal drugs. It has high accuracy, reproducibility and are able to document the results when compared with TLC (Mahesh attimarad *et al.*, 2011) [24].

The use of fluoroquinolones in food-producing animals has generated a problem of microbial resistance and allergies (Lolo *et al.*, 2005) [23]. The presence of antibiotic resistance in the food-producing animal has gained attention from national and international public health agencies (Salehzadeh *et al.*, 2006) [29]. Djamartumpal and Lumban Batu (2010) [8] reported a method for the determination of oxytetracycline in rainbow trout muscle, liver, kidney, and blood using HPLC with ultraviolet detection. The reported limit of detection was 0.05 ppm for muscle, 0.1 ppm for liver and kidney and the reported recoveries of OTC from blood, liver, muscle, and kidney of rainbow trout were 90, 72, 84, and 73 %, respectively which was similar to our result.

A reversed-phase high-performance liquid chromatographic method with tandem mass-spectrometric detection was evolved and proved for the concurrent analysis of eight quinolones and fluoroquinolones. Quite consistent with the results of the current study (Johnston *et al.*, 2002) [14] reported that, this analytical method has good recoveries for all the analytes and the limit of detection was 1-3 µg kg^{-1} which depend on the analyte and matrix. The limit of quantification was 5µg kg^{-1} (10µg kg^{-1} for ciprofloxacin).

High performance liquid chromatographic method with fluorescence detection for the screening and quantification of flumequine, oxolinic acid, and sarafloxacin in fish was developed. The linearity, recovery, accuracy, and precision of the method were assessed from fortified tissue samples at concentration levels ranging from 75-600µg kg^{-1} for oxolinic acid and flumequine and 15-120µg kg^{-1} for sarafloxacin. The limit of detection for sarafloxacin, oxolinic acid and flumequine were 2, 5 and 7 µg kg^{-1}. The limit of quantification for sarafloxacin, oxolinic acid and flumequine were 15 µg kg^{-1} and 75 µg kg^{-1}. Recovery percentage for quinolones in fish ranged from 56.9 to 71.0%, which were quite similar to the current study (Roudat and Yorke, 2002) [28].

A HPTLC – Fluorescent Densitometry Assay was used for the simultaneous detection of enrofloxacin and ciprofloxacin in broiler chicken tissues. The limit of detection for enrofloxacin and ciprofloxacin were 2 and 3 ng/band. The limit of quantification was 5 ng/band for both the compounds. The percentage recovery for enrofloxacin was 83.4-90.3% when compared to ciprofloxacin 82.0-86.8%. (Sureshkumar and Sarathchandra, 2017) [33].

This study has standardized the HPTLC method for detecting antibiotic residues in Carp and Gold fish. Therefore HPTLC which is a simple and easy method can be used for the detection and quantification of antibiotic residues in fish samples.

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