Journal of
Pharmacology & Clinical Research
ISSN: 2473-5574

Research Article
Volume 10 Issue 3 - July 2024
DOI: 10.19080/JPCR.2024.10.555786

J of Pharmacol & Clin Res

Precision and Validation of Thin Layer Chromatography Analytical Technique to Analyze & Monitoring Antibiotic Residues in Poultry Products and Byproducts

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Submission: June 19, 2024; **Published:** July 01 , 2024

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Abstract

Antibiotic therapy has great importance in human and veterinary medicine. However, indiscriminate uses of antibiotics become a matter of concern for antimicrobial residues and resistance. To ensure consumer protection, this work is designed to develop proper mobile phases for precise detection and conclusion of antibiotics residues present in the poultry products and byproducts in Bangladesh. The TLC Silica gel $60F_{254}$ was used as stationary phase. We started the test with a common starting solvent 1:1 hexane: ethyl acetate followed by different ratios of varieties of chemicals and/or combinations of chemicals. We developed best mobile phases for specific antibiotics usually practiced in poultry production in Bangladesh. We established acetone: acetic acid: methanol (1:1:1) for amoxicillin; acetone: acetic acid (1:1) for cephalexin; methanol: acetonitrile: acetic acid (1:1:1) for oxytetracycline; methanol: toluene: acetic acid: acetone (2.5:2:2:2) for chlortetracycline; acetone: methanol (1:1) for ciprofloxacin; acetone: acetic acid: methanol (1:1:1) for enrofloxacin; methanol: acetonitrile: acetic acid (1:1:1) for levofloxacin respectively. After fixing the best mobile phases for each antibiotic, we validated the technique by testing our indoor and field poultry samples. We further investigated whether TLC analysis could be used for quantitative analysis of antibiotic residue in samples. Concentration dependent analysis of samples by TLC demonstrated quantification of antibiotics presence in the samples. Therefore, the TLC technique would bring success to the livestock sector to combat veterinary antibiotics residues and human health hazards.

Keywords: TLC; Mobile Phases; Antibiotic Residue; Antibiotic Resistance

Introduction

Antibiotics are the most widely used veterinary drugs in the poultry industry [1]. They are used by the poultry industry and veterinarians to enhance growth rates, health, feed efficiency, and egg production, or for therapeutic reasons and prophylaxis measures to reduce the incidence of poultry diseases [2,3]. In Bangladesh, a large population of poultry farming is done by illiterate poultry farmers who do not care about veterinary prescription and supervision resulting indiscriminate use of antibiotics in poultry farming. Human consumption of toxic levels of antibiotic residues in poultry origins has caused several pathological defects in humans that are of public health importance [4,5,6]. Therefore, poultry treated with antibiotics is required for a specific withdrawal period until all residues are depleted to safe levels before their tissues can be used for human consumption [7,8].

Nowadays, the appearance of antibiotic residue in edible animal tissues remains a global problem [9]. The people in Bangladesh are still not aware of the antibiotic residues and the health hazards of antibiotic residues. There is no extensive research work on antibiotic residue detection and hazard analysis in Bangladesh. Moreover, there is no specific residue monitoring program in Bangladesh. Without ensuring our edible poultry tissues and products are free of antibiotic residue and micro-organisms through proper research and investigations, we cannot

be able to export our poultry product to earn foreign currency. The project is directly concerned with national development. If we are not aware of the people, in the long run, the nation will face unlimited and irreparable loss. The indiscriminate use of antimicrobials leads to several life-threatening implications. Among them, the most prevalent effects are antimicrobial resistance, and antibiotic residues in food and animal products which cause hypersensitivity reactions, alteration of gut microflora, and residual toxicity. Chromatographic techniques are preferred to other analytical techniques even though they are quite expensive and sophisticated. Thin-layer chromatography is one of the cheaper and reliable analytical techniques for antibiotic residue analysisII [10]. The goal of TLC is to obtain well-defined, well-separated spots. TLC uses a stationary phase, usually alumina or silica, which is highly polar (standard) or non-polar (reverse phase), and a mobile phase, some solvent whose polarity will be chosen. In most cases, silica plates would be used. Standard and test substances are then applied to the plate and then "run" the plate by allowing a solvent (or combination of solvents) to move up the plate by capillary action [11].

Depending on the polarity of the components of the mixture, different compounds will travel different distances up the plate. More polar compounds will "stick" to the polar silica gel and travel short distances on the plate, while non-polar substances will diffuse into the solvent and travel large distances on the plate. The measure of the distance a compound travels is called R_f. This number, between zero and one, is determined by measuring the distance the compound moved from the baseline (where it was originally spotted) divided by the distance the solvent moved from the baseline. Determine the solvent system. Compounds will travel different distances up the plate depending on the solvent used. In non-polar solvents like pentane and hexane, most polar compounds will not move, while non-polar compounds will travel some distance up the plate. In contrast, polar solvents will usually move non-polar compounds to the solvent front and push the polar compounds off of the baseline [12]. A good solvent system moves all components of your mixture off the baseline, but does not put anything on the solvent front-R_f values between 0.15 and 0.85. This is not always possible but should be the goal when running a TLC. Silica gel is used and its chromatographic mechanism is adsorption. Therefore, farmers' awareness, residue detection tools availability, and surveillance & monitoring systems are unavoidable in controlling of antibiotic residues in poultry products and byproducts.

Methodology

Study design

A preliminary survey on poultry farming was made in the north area (Dinajpur District) of Bangladesh and some commercial farms were selected for the study after making a contract with the farmers. A meeting was held in each farm to obtain data on the rearing system, treatment records, name & type of drugs used, withdrawal period maintenance, etc. An awareness campaign was also held on each farm to teach the poultry farmers about the residual effects of antibiotics and hazards to human health. After a successful discussion, necessary samples (liver, thigh muscles, and breast muscles) were collected for laboratory investigation [13].

Screening of antibiotic residues

The experiment was conducted in the Department of Pharmacology, Bangladesh Agricultural University (BAU), Mymensingh by TLC technique. All chemicals used were analytical grade.

Thin Layer Chromatography

TLC is a sensitive and exact-reliable method for monitoring low amounts of different biological chemicals. Illumination of antibiotics against UV light helps as a simple detector for this mean. Employment of thin-layer chromatography (TLC) in pharmaceutical and medical clinical or biological research comprises more than 50% of the technique's total application. Determination of drug residues in food is an important application of TLC.

Principle: Like other chromatographic techniques, TLC depends on the separation principle. The separation relies on the relative affinity of compounds towards both phases. The compounds in the mobile phase move over the surface of the stationary phase. The movement occurs in such a way that the compounds that have a higher affinity to the stationary phase move slowly while the other compounds travel fast. Therefore, the separation of the residues is attained. On completion of the separation process, the individual components from the mixture appear as spots at respective levels on the plates. Their character and nature are identified by suitable detection techniques.

TLC apparatus

TLC plate (MN-Germany), TLC tank, and UV detection box (UV light: F18W-Germany), were used. TLC was performed and the same R_f value of standard and sample considered similar compound.

Materials and reagents

Beaker, Pipette, Micropipette, Falcon tube, Capillary tube, Mortar and Pestle, Measuring tube, Electronic Weigh machine, Vortex machine, Food grinder, Centrifuge Machine, Eppendorf tube, Tips, Scissors, Pencil, Scale, Methanol, Acetonitrile, Trichloroacetic acid, Diethyl ether, Distilled water.

Preparation of TLC-Silica Plate

TLC Silica gel 60 F_{254} (Merck, KGaA, 64271 Darmstadt, Germany), was used for TLC analyses.

Preparation of Standard Solution

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Standard was prepared with routinely used antibiotics (Amoxicillin, Cephalexin, Oxytetracycline, Cholortetracycline, Ciprofloxacin, Enrofloxacin, and Levofloxacin).

Mobile phase analysis and selection:

Proper solvent selection is the most important aspect of TLC, and determining the best solvent may require a degree of trial and error. As with plate selection, keep in mind the chemical properties of the analytes. A common starting solvent is 1:1 hexane: ethyl acetate. Varying the ratio can have a pronounced effect on R_f. R_f values range from 0 to 1 with 0 indicating that the solvent polarity is very low and 1 indicating that the solvent polarity is very high. When experimenting, do not want your values to be 0 or 1 because components that are separating have different polarities. If the value is 0 need to increase your solvent polarity because the sample is not moving and sticking to the stationary phase. If the value is 1, we need to decrease solvent polarity because the compound was not able to separate. Acids, bases, and strongly polar compounds often produce streaks rather than spots in neutral solvents. Adding a few percent of acetic to the solvent can correct streaking with acids.

For polar compounds adding a few percent methanol can also improve results. The volatility of solvents should also be considered when chemical stains are to be used. Any solvent left on the plate may react with the stain and conceal spots. Many solvents can be removed by allowing them to sit on the bench for a few minutes, but very nonvolatile solvents may require time in a vacuum chamber. Volatile solvents should only be used once. If the mobile phase is used repeatedly, results will not be consistent or reproducible. The purity of solvents and quantity of solvent mixed should be strictly controlled. Till now, we found the best TLC mobile phase for Amoxicillin was hexane: ethylacetate, 1:1; for Cephalexin was acetone: methanol, 1:1; for Oxytetracycline was butanol: DW: AA, 6:2:2; for Chlortetracycline was butanol: DW: AA, 6:2:2; for Ciprofloxacin was butanol: DW: AA, 6:2:2; for Enrofloxacin was hexane: ethyl acetate, 1:1 and for Levofloxacin was hexane: ethylacetate, 1:1 respectively.

Spotting and running of TLC plate

a) TLC plate was cut into appropriate size (5x4 cm)

b) A straight line was drawn across the plate approximately 2cm from the bottom by a pencil. Another straight line was drawn across the plate below 1cm from the upper edge of the plate.

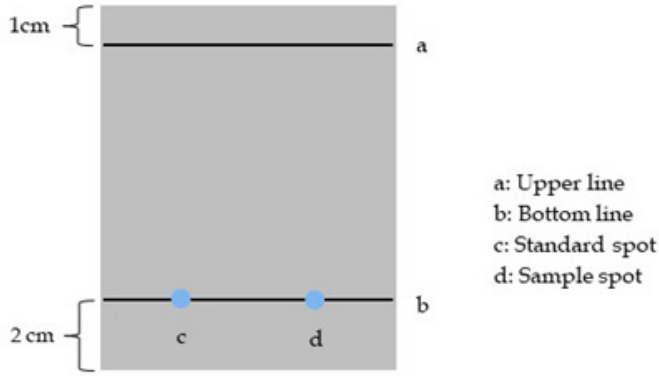
c) Desired spots marking were marked on the bottom line where analytes were dropped.

d) Spots were applied to the plate using thin capillary glass pipettes. A volume of 10µl was used for spotting.

e) Plate was placed in TLC tank containing mobile phases (described in table) and covered by lid and it was left until the mobile phase reached the upper line.

f) Spots were visualized in UV detection box at 256 nm and 365 nm respectively.

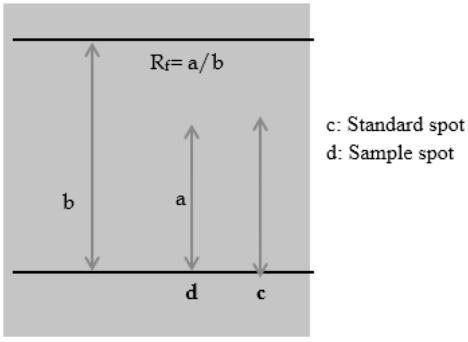
g) Spots marking were done by pencil for calculation of retention factor (R_f)



Calculation of Rf values:

These measurements are the distance travelled by the solvent, and the distance travelled by individual spots.

$$Rf = \frac{Distance\ travelled\ by\ the\ sample\ (a)}{Distance\ travelled\ by\ the\ solvent\ (b)}$$



a: Distance travelled by sample
b: Distance travelled by solvent

Interpretation of results

In order to interpret the results, first setting of standard with reference/pure substances was determined with three repeated times of examination by standard solution. A substance was positively identified in the unknown solution when it behaved identically as the reference substance. That is, after comparison of two substances (standard & unknown) based on following criteria a sample was positively identified such as:

i. Same color under UV light

ii. Same R_f value as those of the reference sample

The components, visible as separated spots are identified by comparing the distances they have traveled with those of the known reference materials. Measure the distance of the start line to the solvent front. Then measure the distance of center of the spot to the start line. Divide the distance the solvent moved by the distance the individual spot moved. The resulting ratio is called R_f-value. As the chemicals being separated may be colorless,

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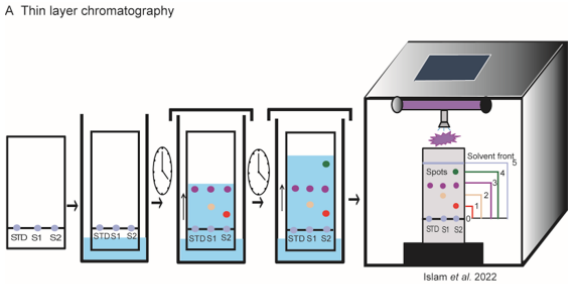
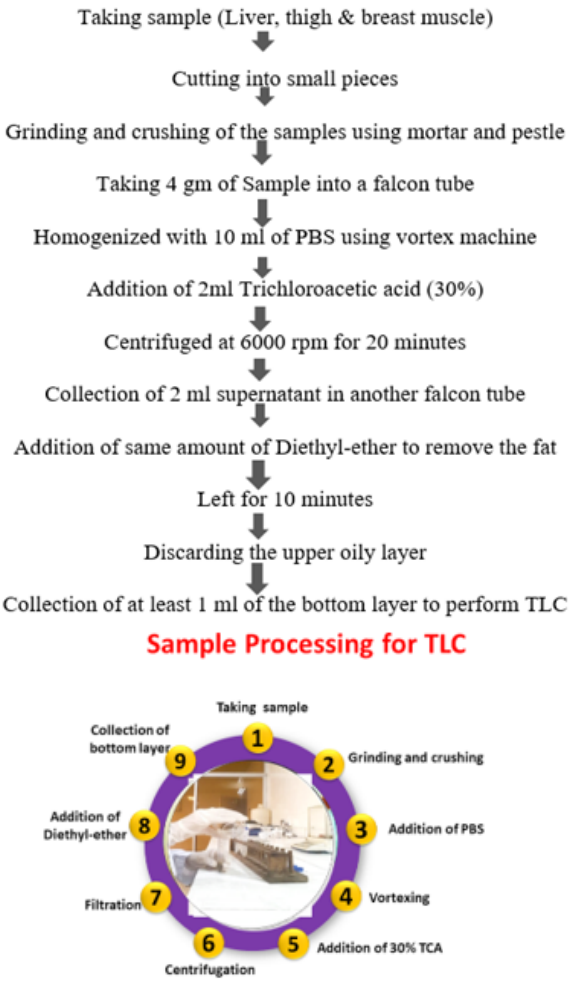
several methods exist to visualize the spots. Often a small amount of a fluorescent compound, usually manganese-activated zinc silicate, is added to the adsorbent that allows the visualization of spots under a backlight (UV_{254}). The adsorbent layer will thus fluorescence light green by itself, but spots of analytes quench this fluorescence, Iodine vapors are a general unspecific color reagent, Specific color reagents exist into which the TLC plate is dipped or which are sprayed onto the plate. Once visible, the R_f value, or retention factor, of each spot can be determined by dividing the distance traveled by the product by the total distance traveled by the solvent (the solvent front). These values depend on the solvent used, and the type of TLC plate, and are not physical constants.



Thin layer chromatographic analysis

Sample preparation and antibiotic extraction

For the detection of antibiotics by Thin Layer Chromatography, these samples were stored in deep freeze at -20°C until further advanced procedures were performed. Samples (liver, thigh muscle, breast muscle) were blended with a mortar and pestle until tissues were mashed properly. These samples were taken into properly cleaned and sterilized Petri dishes with proper care as well as covering. From this 4 g of sample was taken into the beaker with the help of an electric balance and spatula. Then homogenization was done with the addition of 10 ml phosphate buffer (pH 7.2). After proper mixing, the protein contents of these samples were precipitated with the addition of 2 ml Trichloroacetic acid (30%) maintaining sufficient care and attention. Then the mixed samples were taken into properly cleaned and sterilized centrifuge tubes for centrifugation. Then centrifugation was performed at 6000 rpm for 20 minutes with the help of an automatically time-regulated centrifuge machine. The supernatant was extracted with an equal volume of diethyl ether and mixed properly to perform de-fatation. Then the mixture was kept for 10 minutes to separate layers, an upper oily layer and a bottom layer. Then these mixtures were separated from each other. After discarding the upper oily layer, only the bottom layer was collected. This extraction of supernatant was repeated twice with diethyl ether. The extracts were evaporated until dry. Then, extracts were collected into screw-capped vials with proper care and kept in the refrigerator for further advanced analysis. The total procedure was performed as the reference cited by (Popelka et al., 2005).



Sample preparation and antibiotic extraction procedure

Data analysis

Standard statistical procedures were applied in the analysis of data obtained from experiments and analysis

Results and Discussion

We started the test with a common starting solvent 1:1 hexane: ethyl acetate followed by different ratios of varieties of chemicals and/or combinations of chemicals. After preliminary testing and trial for any antibiotics, we have established the best stationary phase and mobile phases for the specific antibiotics analysis and accordingly, we have tested our surveyed samples (detail described in methods and materials). We found the best TLC mobile phase for Amoxicillin was Acetone: Acetic acid: Methanol (1:1:1); for Cephalexin was Acetone: Acetic acid (1:1); for Oxytetracycline Methanol: Acetonitrile: Acetic acid (1:1:1); for Chlortetracycline was Methanol: Toluene: Acetic acid: Acetone (2.5:2:2:2); for Ciprofloxacin was Acetone: Methanol (1:1); for Enrofloxacin was Acetone: AA: Methanol(1:1:1);

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and for Levofloxacin was Methanol: Acetonitrile: AA(1:1:1) respectively. After fixing the best mobile and stationary phase for each antibiotic, we investigated both fields as well as indoor experimental samples with our modified TLC technique. The modified TLC technique was found very valid and effective tool for antibiotic residue detection in poultry samples such as liver, thigh muscle, breast muscle, kidney, etc. Therefore, it could be concluded that the established *De Novo* mobile phages of the TLC technique would bring success to the livestock sector to combat veterinary antibiotics residues and human health hazards.

Survey work was undertaken in the northern area of Bangladesh to make aware of the people concerned with the poultry farming systems. Awareness campaigns were monitored with the theme of indiscriminate use of antibiotics in poultry industries having human health hazards. All poultry farmers were found highly conscious of the danger of antibiotic residues for human consumption. Farmers in the surveyed areas confessed they don't use antibiotics after 20 days of the age of broiler and if used they maintain a withdrawal period before selling for human consumption. Moreover, they shared their practical knowledge on broiler rearing and mentioned that after/or at the age of 20 days broiler birds, don't need any antibiotics treatment. Most of the antibiotics they used in the early life of broilers as disease prevalence in broilers is more before the age of twenty days. Firstly, we have developed the best mobile phase for specific antibiotics (Figure 1). The screening was done with our newly established TLC mobile phases. Till now, we found the best TLC mobile phase for Amoxicillin was hexane: ethyl acetate, 1:1; for Cephalexin was acetone: methanol, 1:1; for Oxytetracycline was butanol: DW: AA, 6:2:2; for Chlortetracycline was butanol: DW: AA, 6:2:2; for Ciprofloxacin was butanol: DW: AA, 6:2:2; for Enrofloxacin was hexane: ethyl acetate, 1:1 and for Levofloxacin was hexane: ethyl acetate, 1:1 respectively.

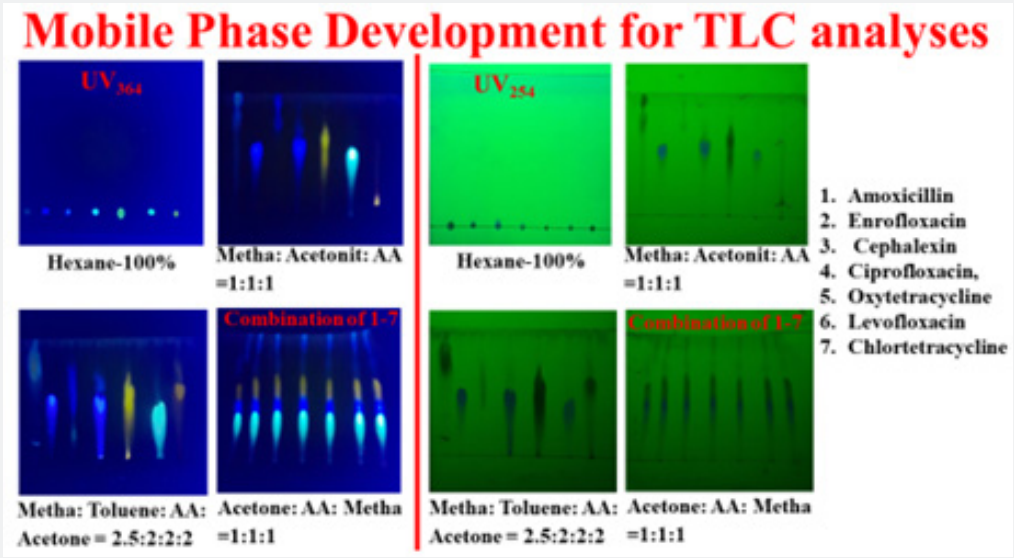

Figure 1: Best mobile phase development analyses. Mobile phases and tested antibiotics were applied to develop the best mobile phase for the specific antibiotic.

A total of one hundred livers, one hundred thigh muscles, and one hundred breast muscles were collected from different farms, market places and analyzed in the Pharmacology Laboratory, Bangladesh Agricultural University, Mymensingh, Bangladesh. The antibiotics screening test was done on the very popular & mostly used antibiotics namely amoxicillin, cephalexin, oxytetracycline, cholortetracycline, ciprofloxacin, enrofloxacin and levofloxacin. Results were demonstrated in Table 1 and Figure 2-4 respectively. TLC analysis revealed that Out of one hundred liver, one hundred thigh muscle, and one hundred breast muscle samples respectively; seven liver, four breast muscle, and four thigh muscle samples were found positive for amoxicillin antibiotic; five liver, three breast muscle, and three thigh muscle samples were found positive for cephalexin, four liver, two breast muscle, and two thigh muscle samples were found positive for oxytetracycline; five liver, four breast muscle, and four thigh muscle samples were found positive chlortetracycline; six liver, five breast muscle, and five thigh muscle samples were found positive for ciprofloxacin; three liver, two breast muscle, and two thigh muscle samples were found positive for enrofloxacin; three liver, three breast muscle, and three thigh muscle samples were found positive for levofloxacin.

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Table 1: Antibiotics residues.

Name of samples	Liver, n=100 Prevalence %	Thigh muscles n=100 Prevalence %	Breast muscles n=100 Prevalence %	R_f value
Amoxicillin	7%	4%	4%	Same with std.
Cephalexin	5%	3%	3%	Same with std.
Oxytetracycline	4%	2%	2%	Same with std.
Chlortetracycline	5%	4%	4%	Same with std.
Ciprofloxacin	6%	5%	5%	Same with std.
Enrofloxacin	3%	2%	2%	Same with std.
Levofloxacin	3%	3%	3%	Same with std.

Mobile phases

Till now, we found the best TLC mobile phase for Amoxicillin was hexane: ethylacetate, 1:1; for Cephalexin was acetone: methanol, 1:1; for Oxytetracycline was butanol: DW: AA, 6:2:2; for Chlortetracycline was butanol: DW: AA, 6:2:2; for Ciprofloxacin was butanol: DW: AA, 6:2:2; for Enrofloxacin was hexane: ethyl acetate, 1:1 and for Levofloxacin was hexane: ethylacetate, 1:1 respectively.

Detected Antibiotics	Stationary phase	Mobile Phase	Fundamental absorption band of antibiotics l=254	Fundamental absorption band of antibiotics l=365	Remarks
Amoxycillin	TLC Silica gel $60F_{254}$	**1:1 hexane:ethyl acetate**	Best		Satisfactory
		1:0 Hexane	-	-	-
		1:1 acetone: methanol	-	-	-
		6:2:2 butanol:DW:AA	-	-	-
		1:1 acetone: AA	-	-	-
Cephalexin	Do	1:1 hexane:ethyl acetate	-	-	-
		1:0 Hexane	-	-	-
		1:1 acetone: methanol	Best	-	Satisfactory
		6:2:2 butanol:DW:AA	-	-	-
		1:1 acetone: AA	-	-	-
Oxytetracycline	Do	1:1 hexane:ethyl acetate	-	-	-
		1:0 Hexane	-	-	-
		1:1 acetone: methanol	-	-	-
		6:2:2 butanol:DW:AA	Best	-	Satisfactory
		1:1 acetone: AA	-	-	-
Chlortetracycline	Do	1:1 hexane:ethyl acetate	-	-	-
		1:0 Hexane	-	-	-
		1:1 acetone: methanol	-	-	-
		6:2:2 butanol:DW:AA	Best	-	Satisfactory
		1:1 acetone: AA	-	-	-
Ciprofloxacin	Do	1:1 hexane:ethyl acetate	-	-	-
		1:0 Hexane	-	-	-
		1:1 acetone: methanol	-	-	-
		6:2:2 butanol:DW:AA	Best	-	Satisfactory
		1:1 acetone: AA		-	
Enrofloxcacin	Do	**1:1 hexane:ethyl acetate**	Best	-	Satisfactory
		1:0 Hexane	-	-	-
		1:1 acetone: methanol	-	-	-
		6:2:2 butanol:DW:AA	-	-	-
		1:1 acetone: AA	-	-	-

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Levofloxacin	Do	**1:1 hexane:ethyl acetate**	Best	-	Satisfactory
		1:0 Hexane	-	-	-
		1:1 acetone: methanol	-	-	-
		6:2:2 butanol:DW:AA	-	-	-
		1:1 acetone: AA	-	-	-

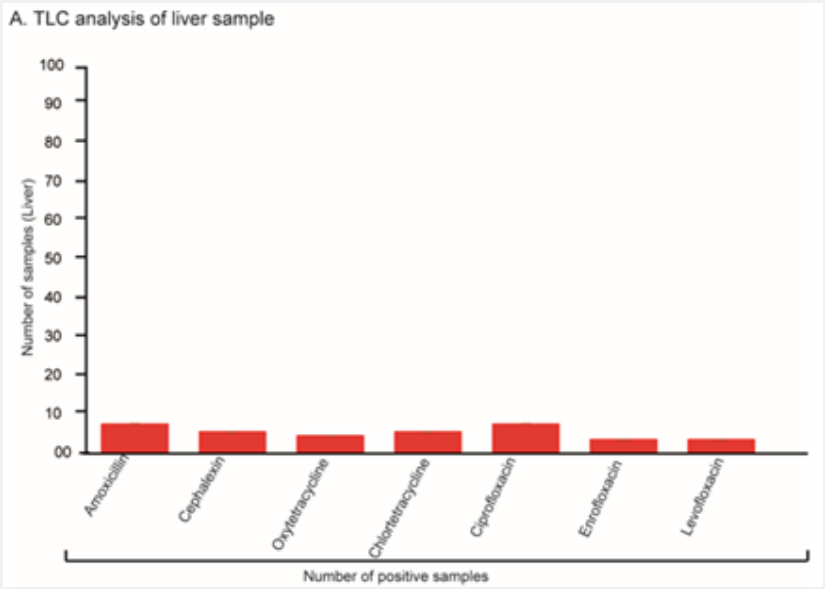

Figure 2: Antibiotics residues in liver. A, demonstrates positive antibiotic residues for amoxicillin, cephalexin, oxytetracycline, cholortetracycline, ciprofloxacin, enrofloxacin and levofloxacin n=100.

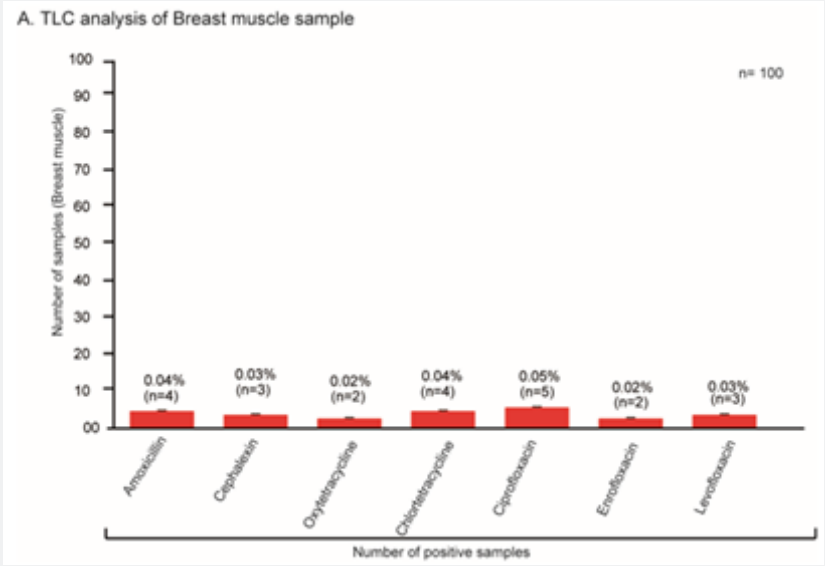

Figure 3: Antibiotics residues in breast muscle. A, demonstrates positive antibiotic residues for amoxicillin, cephalexin, oxytetracycline, cholortetracycline, ciprofloxacin, enrofloxacin and levofloxacin n=100.

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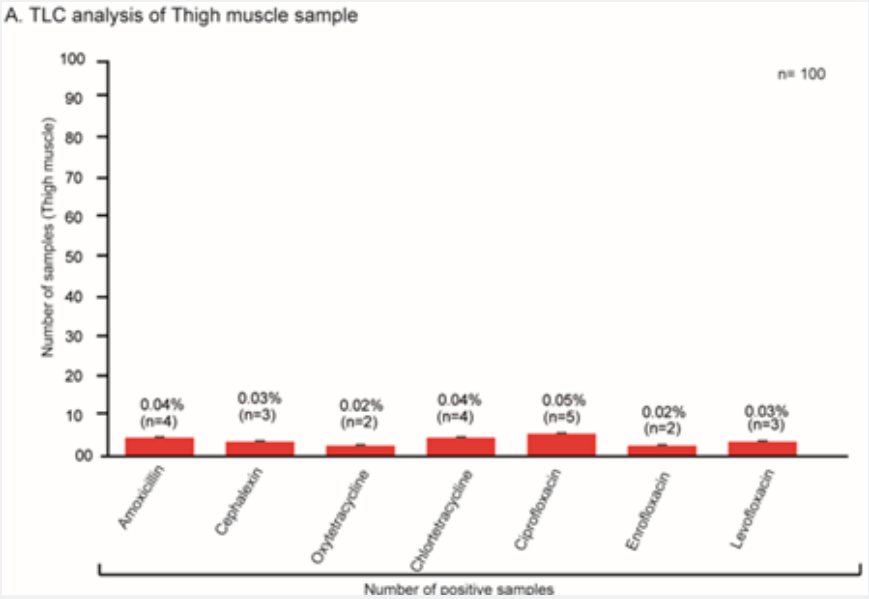

Figure 4: Antibiotics residues in thigh muscle. A, demonstrates positive antibiotic residues for amoxicillin, cephalexin, oxytetracycline, cholortetracycline, ciprofloxacin, enrofloxacin and levofloxacin n=100.

Conclusion

This study was conducted in the Department of Pharmacology, Bangladesh Agricultural University, Mymensingh to establish the de novo mobile phases of the antibiotic TLC technique and to evaluate the antibiotics residues in edible poultry tissues in broilers. Firstly, we have used several mobile phases for the same antibiotics and finally chose the best one for the detection and analysis of specific antibiotics by our newly innovated TLC. A preliminary survey was conducted on poultry farms in the northern area of Bangladesh with the theme of awareness against illegal or indiscriminate use of antibiotics in poultry farms. Farmers were found very aware of the indiscriminate use of antibiotics and the human health hazards of antibiotic residues; only a negligible number of samples were found to be positive for antibiotic residues. Therefore, the innovative TLC technique is a very useful tool to combat the illegal use of antibiotics in poultry farms and to save human health as well.

Funding

This research work was supported by The Bangladesh Agricultural University Research System (BAURES) by a grant in research 2021/89/BAU.

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How to cite this article: Md. Shakil I, Md. Shafiqul I, Md. Rakibul H. Precision and Validation of Thin Layer Chromatography Analytical Technique to Analyze & Monitoring Antibiotic Residues in Poultry Products and Byproducts. J of Pharmacol & Clin Res. 2024; 10(3): 555786.
DOI: 10.19080/JPCR.2024.09.555786

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