ESTIMATION AND QUANTIFICATION OF ANTIMICROBIAL RESIDUE IN POULTRY MEAT AVAILABLE IN AND AROUND JAMMU REGION

by

Lenesha Manhas
(J-19-MV-597)

Thesis submitted to

Faculty of Veterinary Sciences and Animal Husbandry in partial fulfillment of the requirements for the degree of

MASTER OF VETERINARY SCIENCE

IN

VETERINARY PHARMACOLOGY AND TOXICOLOGY

Division of Veterinary Pharmacology and Toxicology

Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu
Main Campus, Chatha, Jammu 180009

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CERTIFICATE – I

This is to certify that the thesis entitled **"Estimation and Quantification of antimicrobial residue in poultry meat available in and around Jammu region"** submitted in partial fulfillment of the requirements for the degree of **Masters in Veterinary Science,** in **Veterinary Pharmacology & Toxicology** to the **Faculty of Veterinary Science,** Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, is original work and has similarities with published work not more than minor similarities as per UGC norms of 2018 adopted by the University. Further the level of minor similarities has been declared after checking the manuscript with URKUND software provided by the University.

The work has been carried out by **Ms. Lenesha Manhas** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma. It is further certified that help and assistance received during the course of thesis investigation have been duly acknowledged.

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Date : 18.10.2021

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CERTIFICATE – II

We, the members of Advisory committee of **Ms. Lenesha Manhas,** Registration No. **J-19-MV-597,** a candidate for the degree of **Masters in Veterinary Sciences** in **Veterinary Pharmacology and Toxicology,** have gone through the manuscript of the thesis entitled **"Estimation and Quantification of antimicrobial residue in poultry meat available in and around Jammu region"** and recommend that it may be submitted by the student in partial fulfillment of the requirements for the degree.

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Date: 18.10.2021

ABSTRACT

Title of the Thesis	:	**Estimation and Quantification of antimicrobial residue in poultry meat available in and around Jammu region**
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Major Subject	:	Veterinary Pharmacology & Toxicology
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Abstract

The antimicrobials like sulphaquinoxaline (SQX) and amprolium (AMP) are used indiscriminately in the poultry sector in Jammu region, J&K (UT). Therefore, the present study was conducted to quantitate SQX and AMP residue status in chicken tissue. Altogether, 20 samples each, collected from Jammu, Kathua, Reasi, Samba, Udhampur for residue analysis of SQX and 15 samples each from all except Udhampur for AMP residues estimation. Each sample included liver, kidney, and muscles. The RP HPLC UV method was used to estimate SQX and AMP residues at 266 and 263nm, respectively. The mobile phase was 0.01M ammonium acetate (pH 4.6), acetonitrile (43:57), flow at 1ml/minutes, peak Rt at 3.813 min. Recoveries of SQX from the liver, muscle, and kidney of chicken, spiked at 0.3 µg/g were 77.484 ± 0.313, 86.838 ± 0.050 and 78.900 ± 0.134%, respectively. LOD and LOQ for SQX were 15.427 ng/ml and 46.749 ng/ml, respectively. The value for r^2 was 0.999, showed good linearity. AMP was analyzed using phosphate buffer and acetonitrile (30:70; v/v) as mobile phase with peak Rt at 2.663min. Recoveries of AMP from the liver, muscle, and kidney of chicken, spiked at 400 ng/g were 72.156±0.704, 71.029±0.575, 68.316±1.495%, respectively. LOD and LOQ for amprolium were 145 ng/ml and 441 ng/ml, respectively. The r^2=0.999 exhibited good linearity. Out of the samples collected, 70% of the liver, 10% of kidney, and 25% of muscles from district Jammu had residue levels beyond established MRL. All the liver samples from Udhampur had deposition of SQX beyond the established MRL. Most tissue samples were positive; however, 50%, 25%, and 45% of the liver samples from Samba, Reasi, and Kathua, respectively, were found SQX residue beyond the prescribed MRL. Most of the tissue samples collected from various districts of Jammu were positive; however, none of the tissues exhibited values beyond established international MRL for AMP residue.

Keywords: Sulphaquinoxaline, Amprolium, LOD, LOQ, MRL.

Lenesha Manhas

Signature of Major Advisor **Signature of Student**

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

%	Percent
±	Plus or minus
=	Equal to
℃	Degree Celsius
≥	Greater than or equal to
b.w.	Body weight
r^2	Correlation coefficient
i.e.	That is
Fig.	Figure
MRL	Maximum residue limit
NOEL	No observed effect level
ADI	Acceptable daily intake
SQX	Sulphaquinoxaline
PABA	Para-aminobenzoic acid
AMP	Amprolium
CAC	Codex Alimentarius Commission
ELDU	Extra-label drug use
LC-MS	Liquid chromatography- mass spectrometry
EC	European Commission
WHO	World Health Organization
FAO	Food and Agriculture Organization

FDA	Food and Drug Administration
RP	Reverse Phase
ACN	Acetonitrile
HPLC	High Performance Liquid Chromatography
UV	Ultra Violet
PDA	Photo diode array
HPLC- DAD	High Performance Liquid Chromatography with Diode-Array Detection
RP- HPLC	Reverse Phase High Performance Liquid Chromatography
RSD	Relative Standard Deviation
Rt	Retention time
LC-UV	Liquid Chromatography with ultraviolet detection
g	Gram
ng	Nanogram
μg	Microgram
Kg	Kilogram
LOD	Limit of Detection
LOQ	Limit of Quantitation
SPE	Solid Phase Extraction
ml	Milliliter
min.	Minutes
mg	Milligram

M, mol	Mole
ppm	Parts per million
rpm	Revolutions per minute
HLB SPE cartridge	Hydrophilic - Lipophilic Balance with Solid Phase Extraction cartridge
c.f.	Correction factor
AUC	**A**rea under curve
ANOVA	Analysis of variance
UV/VIS detector	Ultraviolet-visible detector

CHAPTER-I

Introduction

CHAPTER-I

INTRODUCTION

The poultry industry has a significant role in the Indian agricultural system, and it has been on the rise at approximately 8-10% per annum during the first decade of the 21st century (ICRA, 2014). Poultry meat production is growing at over 10 % during the 2nd decade of the 21st century, the second most widely consumed meat globally and accounts for about 36% of global meat production (Conway, 2017). Broiler production has been livelier, with an annual growth rate of 11.44%, which accounts to be 3.725 million tons and provides employment to 4.29 million people in India (Index Mundi, 2015). Poultry production has a vital contribution to the GDP of India, which accounts for about 0.66%, and thus confers to 7.72% of GDP from the livestock sector (Prabakaran, 2014; Rajendra *et al*., 2014). According to the 19th Indian Livestock Census, conducted in 2012, the per capita consumption of broilers meat was 3.35 kg per person per year for the year 2016-17. The total poultry population in India is 851.81 million, with an increase of 16.8% over the previous Census (BAHS, 2019). Poultry contributes to 50.06% of the total meat production in India.

The scenario of the poultry industry in Jammu (J&K)

The annual growth rate of meat production in UT of J&K accounts for 4.8% and stands 13th among the top meat producer states during 2018-19 (BAHS, 2019). The Union territory of Jammu and Kashmir shares 1.13% of the total Indian poultry population. The poultry sector of Jammu and Kashmir (UT) has a compound annual growth rate of 10%. According to the recent report, in UT of J&K, about Rs. Two thousand crores of meat are imported from outside the state every year. To meet the local demand and bridge the gap, poultry farming in UT of J&K is emerging as an important enterprise and is assisting to create income and employment for the rural youths of the state.

The broiler chicken has been developed for table purposes, which gains weight quickly; simultaneously, the birds are vulnerable to various infectious diseases *viz*

Coccidiosis, Salmonellosis, Ranikhet disease, Marek's disease/Fowl paralysis, Enteritis, Typhoid, Hydro pericardium syndrome, and IBD (Infectious Bursal disease). However, the most common infectious pathogen that happens to the broiler bird during its life span is *Eimeria tenella, Eimeria acervulina, Eimeria necatrix, Eimeria maxima,* and *Eimeria brunetti*. Eimeria species of protozoa produce bloody diarrhea in chickens, which (may) adversely affect the growth and production of birds. In some instances, it may lead to massive mortality of the birds as well. It therefore, causes severe economic losses to the poor farmers. In anticipation of such outcomes or more disease loads, farmers go for heavy and injudicious use of antimicrobial/antiparasitic drugs just to prevent and/or treat such infections. The very advent of antibiotics proved a vital tool to control infectious agents and improve feed efficiencies (Engberg *et al.,* 2000). Antibiotics are the product of biological origin, or they can be identical to synthetic/ semi- synthetic products that kill or inhibit microorganisms' growth (Kirbis, 2007). In principle, antibiotics should be used as a remedial measure to destroy the infectious pathogenic microbes. Still, practically, these agents are used at the sub-therapeutic level with a particular objective, like prevention of potential infections, improvement in feed efficiency, and as growth promoters, etc. These antibiotics include a vast number of compounds that can be administered to the birds orally, in feed, and or drinking water based on veterinary prescriptions. In addition, these antibiotics facilitate consumers with the top quality of meat and eggs at affordable prices (Donoghue, 2003). Antibiotics are commonly recommended to poultry farmers to be used at the sub-therapeutic level to suppress the growth and multiplication of infectious pathogenic agents. In due course, due to selection pressure, the microbes develop resistance (Kolar *et al*., 2001). There starts a vicious circle, and the farmers adopt high dose rates to counter the microbes' associated symptoms and thus leads to rampant and injudicious uses of such antibiotics. This practice of using antibiotics at higher dose rate in birds /animal, the food of animal/bird origin results in the occurrence of its residues that can be transferred to the consumers. This practice can also transmit the pathogenic microbes to the environment, and may affect the consumer by inducing allergy. In addition, this can ship antibiotic-resistant microbial pathogens as well. The antimicrobial drugs used in animals/birds, when appears along with their metabolites associated impurities in food of animal origin, are

called antimicrobial residues. It may lead to severe consequences in consumers if consumed over maximum residue limits for the specific antimicrobial agents. Residues are defined as "all active metabolites of these components that remain in meat or other foodstuffs from the animal origin to which the medicinal product in question has been administered" (EC, 2002). European Parliament, vide Registration No. 470/2009 and the European Council defined residues as "all the pharmacologically active substances, whether active ingredients, excipients or degradation products and their metabolites which remain in animal-derived food." The very concept of antibiotics/drug residues in food of animal origin appeared and developed during the later half of the twentieth century. This also led to the development of some of the associated terminologies, *viz*. NOEL (No observed effect level), ADI (Acceptable daily intake), and MRL (Maximum residue limit) pertaining to the food of animal origin (CAC, 2011). Residues may happen/occur due to some or other reasons like overlooking of withdrawal period for the specific drugs, extra-label uses for animals, and or contamination of animal feed or drinking water, inadvertently by excreta of treated animals or else the use of antibiotics not licensed for (Paige, 1994).

Withdrawal period refers to the duration between the last doses of the drug, administered to the animal/bird, to the level of residues in the tissues and other food products of animal origin, becomes lesser than or equal to the MRL. Until the withdrawal period has elapsed, the animal or its products must not be used for human consumption (Jackson, 1990).

Maximum residue limit (MRL) or tolerance refers to "The maximum concentration of residue resulting from the use of a veterinary medicinal product expressed (μg/kg) on a fresh weight basis which may be recognized by the Community to be legally permitted or recognized as acceptable in or on a food" (Council Regulation EEC 2377/90). The MRL is the target concentration in a residue depletion study. It should be established based on safety to the persons consuming the product and has no pharmacodynamics reality in the animal to which the drug has been administered. Tissue tolerances are normally established in fat, milk, muscle, liver, kidney, skin, or sometimes meat by-products.

The local poultry farmers in the Jammu, J&K (UT) use antimicrobials as disease prophylaxis and growth promoter for quicker growth of chickens within the 4-6 weeks' time, to improve feed assimilation and reduce the mortality due to infectious microbes in intensive rearing. It was observed that sulphaquinoxaline (SQX) and amprolium (AMP) are the commonest antimicrobials, which are used orally via feed and or drinking water in poultry in the Jammu area, primarily for control and prophylaxis of coccidia, and some susceptible bacterial diseases in poultry, and growth promotor as well. However, the data regarding the SQX and AMP residue in poultry, sold in Jammu is very limited and could not be traced. Keeping the view and concerns mentioned above, the study was designed with the following objective:

1. Detection and quantification of antibiotics (with special reference to presence of sulphaquinoxaline and amprolium) in poultry meat sold in and around Jammu region.

CHAPTER-II

Review of Literature

CHAPTER-II

REVIEW OF LITERATURE

"Any substance administered to food-producing animals for diagnostic, therapeutic, or prophylactic purposes as well as for modification of physiological functions or behavior are considered as Veterinary drugs" (Jensen *et al.,* 2003). These drugs are commonly used all over the globe and includes a diversified variety of substances, consist of vaccines, antibiotics/ antimicrobials, anti-parasitic, and β-agonists.

Antimicrobials/antibiotics are vital and commonly used veterinary drugs. Antimicrobials are drugs of natural/ synthetic or semi-synthetic origin that impedes the growth and multiplication of or kills/ or destroys microbes, once applied at low level/concentration without rendering any damage to the host (Prajwal *et al.,* 2017). There are various groups of antimicrobials *viz*. sulfonamides, amprolium, penicillin, streptomycin, tetracyclines, tylosin, aminoglycosides, β-lactams, macrolides, lincosamides, and quinolones that are administered in among bovine, ovine, caprine, swine and poultry production (Alhaji *et al.,* 2018). In addition of antimicrobials, there are some antiparasitic drugs, which are used in livestock production and management, *i.e.,* nitroimidazoles, nitrofurans, coccidiostats, anthelmintics, carbamates, pyrethroids (Prajwal *et al.,* 2017).

Residues are the xenobiotic compounds prevailing in food materials obtained from animal/birds' origin as a result of administration of the chemical that is there in the animal/bird tissue but that are not normally anticipated to be available in it or the compound that exists in high level than usual, plus its active/ inactive metabolites, or any additional compound synthesized thereof, in food as a result of administration of the compound/s (El Kholy & Kemppainen, 2005). "Residues of veterinary drugs include the parent compounds and/ or their metabolites in any edible portion of the animal product and include associated impurities of the concerned veterinary drug" (FAO/WHO, 2008). Veterinary drug residues refers to the presence of antimicrobials or their toxic metabolites prevailing in food of animal origin *viz.,* animal tissues, milk, milk products and eggs of food-producing animals/birds. (Liu, 2011).

Uses of veterinary drugs

Veterinary drugs are commonly used in livestock and poultry for prophylactic and therapeutic purposes thereby, play vital role in poultry production. Veterinary drugs comprise of various types of xenobiotic compounds that are given through oral route via feed or the potable drinking water. They are administered to avert and check various communicable and non-communicable illnesses, assist in mitigating the stress due to harsh environmental alterations, jab, debeaking, and certain management practices (Kabir *et al*., 2004).

2.1 Risk Factors for Drug Residue Occurrence

2.1.1 Extra-label drug use (ELDU)

Every drug is approved by the regulating agency following the specific protocol, including the way and means of application/ route of administration mentioned on its label. So, when the drug is not used according to the label directions, it is called extra label use. For example, when a drug is approved for one ailment and practically used in some other ailment, for which it was not permitted or the use of treatments at levels beyond endorsed dosages, it is referred to as extra-label drug use. For example, Phenobarbital is a drug allowed for use in human treatment only, administered in dogs and cats to treat a seizure. Similarly, enrofloxacin solution, approved for parenteral application (intravascular / intramascular injection) only, when administered topically, for medication in the ear, is extra-label use of the drug in veterinary medicine.

2.1.2 Improper Withdrawal Time

It refers to the duration for the residues of toxicologic values to attain a safe level as quoted by maximum residue limit/ or tolerance. Depending on the drug, dosage form, and route of administration, the withdrawal time may vary from a couple of hours to many days or weeks. It is the interval necessary between the last administration of the drug to the animal under normal condition of user and the time when treated animal can be slaughtered to produce safe foodstuffs.

2.1.3 Disease status

The status of health of an animal affects the pharmacokinetic profile of a drug administered, which can impact the latency for drug residues. The disease state influences the body's biotransformation system, which ultimately affects the consequent drug metabolism), or in the instances of infection and/or inflammation, causes the drug to cumulate in affected tissues. For example, in cattle with acutely inflamed mastitis quarters, apramycin penetrates in these areas of the body, and concentrations of the drug are observed at ten times over the extent recorded from cows without mastitis (Beyene, 2016).

2. Veterinary drugs of interest

WHO (World Health Organization) and FAO (Food Agriculture Organization) have established a standard to protect human health for ADI (Acceptable daily intake) and MRL (maximum residue limit) in foods of animal origin (FAO and WHO, 1995).

The most common antimicrobials, *viz* sulphaquinoxaline, amprolium, oxytetracycline, enrofloxacin, and gentamicin, are commonly used in the poultry sector in prophylaxis and treatment of certain diseases and used as growth promoter as well. Sulfonamide group of antimicrobials, are primarily used to control coccidia in the poultry sector, including some of the susceptible pathogens. Sulphaquinoxaline is a bacteriostatic antimicrobial, which restricts the folic acid synthesis in bacterial cells. This impedes dihydropteroate synthetase and rivals PABA (para-aminobenzoic acid), thereby the synthesis of folic acid interferes, and ultimately cellular replication is restricted (Hela *et al.,* 2003). Ahmad *et al.,* (2019) has found the withdrawal period of 6-7 days after single dose (50 mg/kg b.w.), oral administration of sulphaquinoxaline. FDA (2016) has established a withdrawal period of ten days for sulphaquinoxaline before the slaughter of the animals. In birds, the most common and feasible route for drug administration is via the oral route, *i.e.,* through feed or drinking water. The value of prescribed MRL (maximum residue limit) for liver, kidney, and muscle is 100µg/kg.

Antimicrobials are essential drugs used to treat and prevent diseases and other purposes, including growth promotion in food-producing animals. However, using small

amounts of antimicrobials over a long period leads to the growth of bacteria that are resistant to the drug's effects, endangering humans who become infected but cannot be treated with routine antibiotic therapy. The more the antimicrobials used, the more likely antimicrobial-resistant to develop among pathogens and commensal bacteria in an exposed population of animals (Hamid, 2012). Two most commonly used veterinary drugs sulphaquinoxaline and amprolium have been selected for the study.

2.2.1 Sulphaquinoxaline

Molecular formula: $C_{14}H_{12}N_4O_2S$

Molar mass: $300.366 gmmol^{-1}$

Melting point: 247.5°c

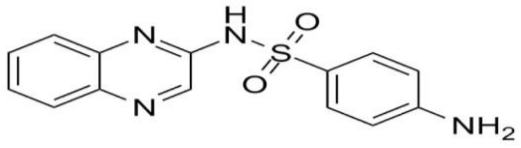


Figure 2.1: Chemical structure of sulphaquinoxaline

2.2.2 Solubility

Slightly soluble in water, ethanol, acetone, and soluble in alkaline solutions. Sulphaquinoxaline is N^1- quinoxaline-2-ylsulphanilamide, and its molecular formula is $C_{14}H_{12}N_4O_2S$. Sulphaquinoxaline is practically insoluble in water, very slightly soluble in alcohol, practically insoluble in ether, but freely soluble in aqueous solutions of alkalis. sulphaquinoxaline is an effective and commonly used coccidiostat throughout the world. It is also effective against *E. acervulina* in addition to *E. necatrix* and *E. tenella*. It exerts marked inhibitory effects on schizogony. This compound has ten days withdrawal premarketing requirement, and eggs from treated birds should not be used for human consumption (FDA, 2016).

2.2.3 Mechanism of Action

It is a member of the sulfonamides, bacteriostatic antimicrobial that interferes with the biogenesis of folic acid in bacterial cells by competitively preventing para-aminobenzoic acid (PABA) from incorporation into dihydrofolic acid. By replacing the PABA molecules in dihydrofolic acid, they prevent the formation of folic acid required for the synthesis of nucleic acid and multiplication of bacterial cells (Prescott *et al*., 1993; Appelgate, 1983).

2.2.4 Pharmacokinetics

Sulphaquinoxaline is available as an oral solution in a range of concentrations (20-32%). These solutions are intended to be mixed with drinking water. For the use in poultry, it has been administered to control coccidiosis. Mathis *et al.,* (1984) described the therapeutic effectiveness of sulphaquinoxaline and sulphaquinoxaline-pyrimethamine against several coccidia species of Eimeria. It was determined from a study that both sulphaquinoxaline and sulphaquinoxaline-pyrimethamine were highly effective against *E. acervulina* but less effective against *E. tenella*. Banerjee *et al.,* (1974) reported on the blood concentrations after administration of sulphaquinoxaline, which were in the therapeutic range. In that same study, sulphaquinoxaline was found in high concentrations in the liver, kidney, and cecum, with the lowest concentrations found in the yolk sac and brain. The time required to reach maximum plasma concentration in broilers for sulphaquinoxaline is of 5.5 hours (El-Sayed *et al.,* 1995)

Sulphaquinoxaline accumulates more rapidly in chicken blood than do the more commonly used sulfonamides. Sulphaquinoxaline diffuses into the egg in proportion to the blood concentration. Acetylation is not extensive in chicken. After the sulphaquinoxaline supply is removed, the concentration in blood decreases slowly, and little traces of the drug are often detected after 112 hours. A study by Li *et al.,* (1995) found that in 7-8 week old male and female broilers given a single 200 mg/kg oral dose of sulphaquinoxaline, peak concentration times in plasma and liver were similar (4 hours) but were longer in the heart, kidney, and muscle (6 hours). The half-life of sulphaquinoxaline was shortest in the muscle (4.5 hours), with significantly longer half-lives in the heart (10 hours), plasma (11 hours), liver (13 hours), and kidney (18 hours).

Sulfonamides are usually easily and extensively metabolized (Kahn *et al*., 2005). They are primarily metabolized in the liver, but they are also bio-transformed in other tissues, e.g., kidney, lung, brain, adrenal, blood, neurons, skin, and gastrointestinal tract (Botsoglou and Fletouris, 2001). Acetylation at N4-position is the major process for most sulfonamides; the other mechanisms of metabolism include aromatic hydroxylation, glucuronidation, O-dealkylation, deamination, and sulfation (Vree *et al.,* 1985). In general, metabolites of sulfonamides are more water-soluble and rapidly eliminated from

the parent drug (Nouws *et al.*, 1988). In poultry, approximately 6-20% of most sulfonamides are metabolized by acetylation (Kietzmann, 1980; Vree and Hekster, 1985; Nouws *et al.,* 1988). The low yield of plasma N4 – acetylsulfonamide may be due to a high rate of deacetylation in chickens (Oikawa *et al.,* 1977), a high fecal excretion rate, or a preferred metabolic pathway by hydroxylation or deamination (Jain, 1994).

Kidneys play the primary route of elimination for most sulfonamides either as parent substance or metabolite; by glomerular filtration (unchanged), active carrier-mediated proximal tubular secretion, and passive reabsorption of nonionized drugs from the distal part of the renal tubules (Prescott, 1993 and 2000; Plumb, 1991 and 2005). Other less significant routes of excretion are via milk, bile, feces, tears, and sweat (Spoo and Rivere, 2001). Enteric sulphonamides are primarily eliminated via the feces, with little of the metabolized drug being excreted by renal mechanisms.

2.2.5 Toxicity

Sulfonamides are relatively non-toxic when used in normal therapeutic doses (Barragry, 1994). Nevertheless, except for the sulfa pyrimidine group (sulfamethazine, Sulfadiazine, and sulfamerazine), sulfonamides are predisposed to precipitation in renal tubules due to the low water solubility of acetylated forms (Riviere *et al.,* 1991; Prescott, 2000). Crystalluria, hematuria, and renal tubule blockage can occur due to the precipitation of the sulfonamide within the glomerular filtrate of the kidney. Some other toxic effects have been documented, e.g., allergies in humans (Choquet- Kastylevsky *et al.,* 2002); keratoconjunctivitis sicca in dogs (Collins *et al.,* 1986), and hepatic necrosis in dogs. Toxicity from sulphaquinoxaline has occurred in Leghorn chickens (Daft *et al.,* 1989), where mortality of 47% was reported in a commercial flock given a 0.05% concentration of sulphaquinoxaline in the feed. Lesions included mildly enlarged livers; swollen and pale livers; hemorrhages on the epicardium, kidney, oviduct, small intestine, and caecum; pale bone marrow; gangrenous dermatitis; and some lung involvement was present. Hypoprothrombinemia has been reported in dogs (Neer and Savant 1992; Patterson and Grenn, 1975), in coyote pups (Brown *et al.,* 1989), and Leghorn chicken (Daft *et al.,* 1989) given sulphaquinoxaline. Sulphaquinoxaline can induce hypoprothrom binemia in animals within 24 hours after dosing by lengthening prothrombin time.

2.2.6 Reviews: Analysis of Sulphaquinoxaline

Takahashi *et al*. (1994) reported the RP-HPLC-UV method to simultaneously quantify sulphaquinoxaline and its major metabolite (N4-acetylsulphaquinoxaline) as well as ethopabate in poultry tissues. The chicken tissues were fortified using 0.1µg/gm of sulphaquinoxaline and achieved recoveries up to 103.8 ± 3.4%, 97.4 ± 5.3%, 99.1±3.3%, 100.1±4.2%, 81.0±6.1% in muscles, liver, kidney, skin, and plasma respectively running the five replicates. In addition, they achieved the limit of detection up to 0.003 µgm/gm.

Simeonidou *et al.* (1996) demonstrated a rapid liquid chromatographic method to quantify some of the sulfonamide antimicrobials residues, including sulphaquinoxaline in chicken tissue. This group extracted sulfonamides with chloroform, HCl and taken up the pre-column derivatization with the help of fluorescamine. The analysis of the derivatized fluorescent product was performed on the C18 column. They devised the mobile phase, which included a 20 mM phosphate buffer: Acetonitrile (66:34, v/v) along with Sodium octane sulfonate (20 mM). They achieved the residues detection limit up to 40 ngm/gm for sulphaquinoxaline in chicken muscles along with recovery up to 82.5%.

Atta *et al.* (1999) studied sulphaquinoxaline residue along with other sulfonamides (Sulfadiazine and sulfadimidine) in apparently healthy, and *E. stiedai* infected rabbits at the dose of 500 mg/liter in drinking water for five days. Sulfadiazine was found in the liver and kidney at relatively lesser concentration than required to be over tolerance level on day 5 of the treatment, although the drug was no more traceable on day seven post-treatment. The concentration traced for sulfadimidine and sulphaquinoxaline was up to day five, and maximum concentrations were 0.08 and 0.09 µgm/gm, respectively, detected in the liver. Although, the picture was a little different in infected rabbits. It was found that all three sulfonamides were traceable up to day seven at over 0.1 µg/gm in the liver and kidney only. Based upon their study, they concluded that the withdrawal period for sulfonamides antimicrobials may be 4-5 days in apparently healthy rabbits and 7-8 days in *E. stiedai* infected rabbits.

Stoev and Michailova (2000) quantified sulphaquinoxaline residues employing derivatization at 60°C for ten minutes, followed by incubation for twenty minutes, before injecting in HPLC for analysis within the pH 2.5-3.5. The mobile phase suited for the purpose was phosphate buffer (pH 3). They achieved the recovery up to 65%, 66%, and 75% employing the spike of 1 µg/Kg, 5 µg/Kg, and 10 µg/Kg sulphaquinoxaline, respectively, which was noticed lowest as compared to other sulfonamides during their study. The respective values for RSD were 22%, 14%, and 15%. The quantitation limit (LOQ) for sulphaquinoxaline was achieved as 1 µg/kg and 0.2 µg/kg for remaining sulfonamides in meat samples. The detection limit for sulfonamides obtained was 0.05 µg/kg.

Kao *et al.* (2001) studied sulphaquinoxaline along with seven other sulfonamides and including drugs commonly used in animals *viz.* ethopabate, furazolidone, carbadox, clopidol, and ormetoprim using HPLC equipped with PDA in swine and poultry muscles. The residue was extracted using acetonitrile as the solvent and filtered, followed by liquid: liquid extraction evaporated to render the extract dry. The residue was passed through the Sep-Pak C18 column, then subjected to HPLC-PDA analysis. Gradient elution was adopted using sodium dihydrogen phosphate: acetonitrile as mobile phase. The group achieved the mean recoveries of the drugs under study ranged from 71.1 to 99.6%, along with achieved limit of detection was 0.04-0.02 ppm. Out of 25 muscles, samples of poultry and pig gathered from local vendors in Taipei, Taiwan, one muscle sample of bird contained 1.23 ppm sulphaquinoxaline, which violated the residue limit.

Khishida and Furusawa (2003) reported an HPLC-PDA method for simultaneous estimation and quantification of six sulfonamides, including sulphaquinoxaline in tissues like chicken, beef, and pork. The sample was homogenized along with alumina, placed in a column, eluted using ten percent of 70% alcohol under gravity. The eluate obtained was allowed to dry, reconstituted in one ml of the mobile phase. The mobile phase was constituted of alcohol and 2% (v/v) aqueous acetic acid of pH 2.7 in the ratio of 25:75 (v/v). The samples were subjected to Mightysil RP-4 GP column and analyzed using a PDA detector. The mean recoveries of the sulphaquinoxaline, in fortified samples obtained, ranged from 91.6 to 97.6% along with quantitation limit, ranged from 0.031 to

0.033 ppm in tissues of animal and or bird origin. The recoveries obtained in the case of other drugs under study were over 85%, and the limit of quantitation for all the sulfonamides under study ranged from 6 to 40 ng/gm in the same sets of animals and or bird tissues.

Salisbury *et al.* (2004) developed a liquid chromatography method for the determination of sulphaquinoxaline, along with other sulfonamides residues in the muscle, liver, and kidney of food animals using sulfapyridine as internal standard. The sulfonamide derivatized products were separated by HPLC using a C18 column, mobile phase 0.02 M phosphoric acid-acetonitrile (60.5:39.5) and detected by fluorescence. The method was applied to swine and cattle muscle, liver, and kidney; sheep and horse muscle and kidney; and chicken muscle and liver. The mean values for samples spiked with sulfonamide at levels between 0.05 and 0.2 μg/g agreed within 96-99% of fortification levels, with coefficients of variation ranged from 4-10%. The limit of detection (LOD) for all sulphonamides achieved was 0.01 μg/gm except for sulphaquinoxaline, for which the LOD obtained was 0.015 μg/gm.

A method was developed by Zhang and Yinliang (2005) for determination of residual sulfonamides including sulphaquinoxaline in tissues of swine and poultry origin using solid-phase extraction (SPE) and HPLC with a photodiode array (PDA) detector. The detection limits (LOD) were 3 μg/kg for most of the sulfonamides under their study. However, the LOD for sulphadimethoxine and sulphaquinoxaline obtained were 7 μg/kg. The quantitation limits (LOQ) were 10 μg/kg for sulphamethazine, sulphamono-methoxine, and sulfamethiazole, and 25 μg/kg for sulfadimethoxine and sulphaquin-oxaline. The recoveries for these antimicrobials attained were in the range of 73.2% to 97.3% on fortification at the level of 50 μg/kg.

Sabatino *et al.* (2007) devised an LC-UV method for concurrent estimation of ten commonly used sulfa antimicrobials, including sulphaquinoxaline. These sulfonamides are commonly utilized in veterinary drugs in cattle, sheep, and birds. This LC-UV method was employed to muscles samples of bovines, poultry, and ovine origin. The targeted residues were hauled out with the use of acetone-chloroform solvent. These

sulphonamides were retained in the solvent used for extraction, followed by elution using methanol: aqueous ammonia solution employing cation exchange SPE cartridge. The eluant was evaporated under liquid nitrogen to dry, again dissolved with methanol (5ml), and sieved (0.45µm), and then injected in an LC-UV system for analysis. The chromatographic separation was achieved using the C18 column along with the guard column and gradient mobile phase. The mobile phase employed for the purpose was composed of acetonitrile: methanol (70: 30, v/v) as mobile phase B and potassium dihydrogen phosphate (10 mM) as A. The cattle muscles samples were spiked at 100 ng/gm (EC, 2010) using sulfonamides, including sulphaquinoxaline. The recovery percentage of sulphaquinoxaline was found to be 67.9% in bovine tissue, which was a little higher with respect to sulfadiazine. The detection limit for sulphaquinoxaline was 14 ng/gm, and the corresponding limit for quantitation was 46 ng/gm. However, the quantitation limit for other sulfonamides achieved was ranged from 9 to 46 ng/gm. The mean recovery was lowest in the case of pork (66.30%), followed by cattle muscle tissues (68.30%), sheep muscles (68.80%), poultry muscles (70.50%), and cattle ground meat (71.50%).

Cheong *et al.* (2010) studied the residue status of the most common four sulfa antimicrobials, including sulphaquinoxaline, used in poultry rearing in eleven states of Malaysia. They quantified the sulfonamides in chicken liver and breast muscles using RP-HPLC at 266 nm wavelength. The residues of sulfonamides detected in the samples gathered ranged between 6-62 ng/gm in breast meat and 8-193 ng/gm in the liver. Though they observed compliance to the maximum residue limit established in Malaysia, one violative sample was found from Johor state which exhibited the residue concentration up to 152 ng/gm of sulphonamides. This finding rendered the workers conclude that sulfa drug exposure to Malaysian consumers was much lower and ranged in between 2-88 ng/gm.

Chitescu *et al.* (2011) developed the HPLC-UV method for the estimation of four common sulfonamide residues in birds' muscles used in poultry practices. The mining of sulfonamides was accomplished using dichloromethane, acetonitrile, and acetone. Chromatographic separation of the sulfa drugs was done employing RP C18 column

using isocratic mobile phase, composed of methyl alcohol and disodium hydrogen phosphate (6%) in the ratio of 25:75 (v/v). The detection limit (LOD) and quantitation limit (LOQ) attained was 6.53 ng/gm and 19.6 ng/gm, respectively, for sulpha-quinoxaline, which was much higher as compared to other sulfonamides under study. The blank samples were spiked using 50, 100, and 150 ng/gm of standard sulfonamides, which yielded average recoveries ranged from 70% to 84%. The recovery achieved for sulphaquinoxaline was 71%, with the value of r^2= 0.98. The mean RSD was 8% for sulphaquinoxaline.

Mehtabuddin *et al.* (2012) attempted to study the residues status of five of the common sulfonamides in chicken meat and eggs. Altogether, 30 samples each of egg and breast meat were gathered from different local vendors and poultry farms at Rawalpindi and Islamabad, Pakistan. Sulfonamides were mined by liquid: liquid extraction from eggs using acetonitrile and n-hexane, albeit acetonitrile and grade one water was used for extraction of sulfonamide from meat samples, followed by SPE, employing Bond elute C18 column. The analyte of interest was eluted using acetonitrile, evaporated, reconstituted in acetonitrile: water (1:1), filtered, and loaded in HPLC-UV system for detection at 265 nm. The chromatographic separation of sulfonamides was achieved using C18 column and mobile phase composed of methanol and 0.01M potassium dihydrogen phosphate buffer (30:70, v/v). The recoveries of the sulfonamides under this study ranged from 82% to 90% in tissue samples spiked by adding 200 ng/gm. The detection limit obtained was 25 ng/gm for eggs and 20 ng/ml for meat and eggs, respectively. Out of the gathered tissues, 23% of meat and 10% of eggs crossed the prescribed MRLs.

Evanthia *et al.* (2012) screened and quantified sulphaquinoxaline, including nine other sulfonamides in tissues of cattle, pig, and poultry origin using HPLC-DAD at 265 nm wavelength. The chromatographic separation was attained on Kromasil, C_{18} column at room temperature. The sulfonamides were mined from tissues employing a solvent mixture composed of HPLC grade water, ethyl acetate, and acetonitrile. The mobile phase used under the gradient program was composed of acetonitrile, methanol, and 0.1% formic acid. Average recoveries attained were relatively good and ranged from 90.1 to

115.1%, including RSD values were less than 10%. The limit of detection attained was 4.9 ng/gm, and the corresponding limit of quantitation was 14.8 ng/gm in cattle tissues for sulphaquinoxaline. The value of detection and quantitation limits for the same drug were 5.5 and 16.5 ng/gm in porcine tissues and 9.2 and 27.6 ng/gm in chicken tissues.

2.3 Amprolium

Chemical formula: $C_{14}H_{19}ClN_4$

Molar mass: 278.78gmol^{-1}

Melting point: 248-249°C

Solubility

Soluble in water, ethanol and methanol and practically insoluble in isopropanol, ethyl acetate and acetonitrile.

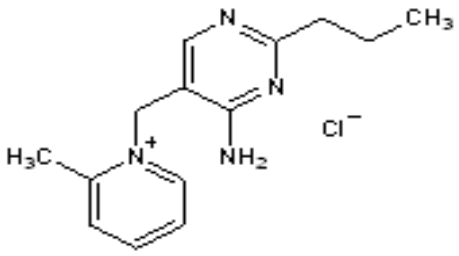


Figure 2.2: Chemical structure of amprolium

Amprolium is 1-[(4-amino-2-propyl-5-pyrimidinyl) methyl]-2-methylpyridinum chloride monohydrochloride) and its molecular formula is $C_{14}H_{19}ClN_4$. It is a coccidiostat that is used for the preemptive action and remedy of coccidiosis in birds, plus laying hens and turkeys as well. It is a structural analog of thiamine (vitamin B_1) but lacks the hydroxyethyl function that thiamine possesses and thus is not phosphorylated to a pyrophosphate analog (Kart and Bilgili 2008). It is a crystalline, odorless, hygroscopic, white powder that is soluble in water and slightly soluble in alcohol.

2.3.1 Mechanism of action

Amprolium is a quaternary compound and is structurally similar to thiamine. Therefore, it mimics the action of thiamine and competitively blocks the thiamine transporter of *Eimeria spp*. By blocking thiamine uptake, it prevents carbohydrate synthesis. This vitamin (thiamine pyrophosphate) is a cofactor of several decarboxylase enzymes, which play a role in cofactor synthesis. It mainly acts on the early first-generation schizonts, preventing the differentiation of merozoites. It may also suppress sexual stages and sporulation of the oocysts.

2.3.2 Antiprotozoal spectrum

It is most active against *Eimeria tenella*, *Eimeria necatrix,* and *Eimeria acervulina* in poultry and has moderate activity against other species of Eimeria. In cattle, it is active against *Eimeria bovis* and *Eimeria zurnii*. In general, it has comparatively less activity against intestinal species than caecal coccidia. It is commonly used along with other anticoccidials (e.g. ethopabate, sulfaquinoxaline, or even pyrimethamine) to improve the spectrum and efficacy against intestinal species of coccidia.

2.3.3 Pharmacokinetics

The drug amprolium is poorly absorbed after oral administration via feed and or drinking water, and is mainly confined to the gastrointestinal tract.

2.3.4 Toxicity

It is a safe drug with a safety margin of about eight times the recommended dosage. In dogs, neurological disturbances, depression, anorexia, and diarrhea have been reported, but the signs are infrequent and are probably dose-related. However, long-term or high-dose administration of amprolium can lead to clinical thiamine deficiency in treated animals, resulting in polioencephalomalacia in sheep and inhibition of erythrocyte production in calves.

2.3.5 Clinical uses

It has been used for the prevention and treatment of coccidiosis in poultry and cattle. It has also been used in dogs, swine, sheep, and goats for the control of coccidiosis. It is an effective prophylactic agent against coccidiosis in chickens, but was not recommended to be used in adult birds. It is the only active pharmaceutical ingredient approved for prevention and treatment in laying chicken. It has a large safety window (at least 5:1) when used at the recommended 125 ppm level in feed (Rychen *et al.,* 2018). It is available commercially as a soluble powder for addition in drinking water or as a premix and is given for prophylaxis to birds at final concentration of 0.0125%. A combination of amprolium and sulphaquinoxaline at levels of 0.006% of each in the food is more effective against poultry coccidiosis than either of the two drugs alone (Long, P.L., 1963). It has zero day pre-slaughter withdrawal time (Sandhu, 2014).

2.3.6 Reviews: Analysis of Amprolium

Nagata and Saeki (1986) developed a method for estimation and quantitation of amprolium residues in the tissue of poultry origin employing LC-post column reaction. Amprolium was extracted from chicken tissues by using methanol and evaporated to 3-4 ml, followed by washing with the help of n-hexane, cleaned up with the help of chromatography using alumina column. The elution of analyte of interest from the interfering matrix was achieved employing SPE using LiChrosorb RP-8 Column, allowed to react with ferricyanide in alkaline milieu, followed by fluorometry at 367 nm and 470 nm wavelength for excitation and emission, respectively. The chicken tissues were spiked by adding 100 and 200 ng/gm amprolium, which led to mean recoveries up to 74.9% and 80.9%, respectively. The limit of detection achieved was 10 ng/gm in chicken tissues and 1ng/gm in standard amprolium.

Leeuwen *et al.,* (1988) developed an incessant current scheme, attached with HPLC for the estimation of amprolium in chicken tissues and egg yolk. The diluted yolk and muscle tissue were extracted using HPLC grade water and dialyzed against grade one water as the receiver stream. The aliquot part of the dialyzed solutions was subjected to the pre-concentration column. The chromatographic separation of amprolium was

achieved using a reversed-phase ion-pair system with the help of post-column oxidation of the drug into amprochrome, which was analyzed with the help of fluorometry. The mean limit of detection obtained was 3 ng/gm. Out of 266 egg (yolk) samples, 29.4% yolk had an average residue of 58 ng/gm amprolium. In comparison, out of 81 muscle samples, only 4.9% had a detectable level of residue with an average concentration of 5 ng/gm.

Hamamoto *et al.,* (1997) devised a quick and sensitive RP-HPLC method for the quantitation of amprolium in the plasma of birds and used it successfully to follow its oral administration in birds. The plasma samples collected from birds were deproteinized by adding 0.33 M perchloric acid, and the top layer or supernatant was taken and injected into the HPLC system using the C18 column. Following the chromatographic separation of beclotiamine and amprolium, the respective derivatized products of beclotiamine and amprolium were synthesized using post-column derivatization followed by analysis using fluorescence detector. This method exhibited good precision, with a detection limit of 2 ng/ml.

Hormazabal and Yndestad (2000) developed a quick and sensitive HPLC method for the estimation and quantitation of amprolium and ethopabate in poultry tissues. The solvent used for chromatographic separation of amprolium as mobile phase was a mixture of solutions A (made by dissolving potassium dihydrogen phosphate: 27.2 g/L plus hexane sulfonic acid sodium salt: 0.94 g/L in 750 ml of HPLC grade water) and B (acetonitrile) (90:10) at the 0.8 ml/minute flow rate. The solvent taken as mobile phase for the chromatographic separation of ethopabate was a combination of acetonitrile: water (35:65) at the flow rate of 0.8 mL/minute. The tissue sample was mined for ethopabate using acetone, followed by separation of the organic layer, and ultimately evaporated to dryness. Chromatographic separation of amprolium was achieved using liquid-liquid extraction from the water phase. The standard curve was linear, with the value of r2=0.9998 for amprolium and 0.999 for ethopabate in poultry tissues. The recoveries percentage achieved for amprolium was ~99% and for amprolium and ethopabate in poultry meat. The quantitation limits obtained were 1 ng/gm and 5 ng/g for ethopabate and amprolium, respectively.

Yamamoto and Kondo (2001) developed an LC method for concurrent quantitation of amprolium and halofuginone in poultry meat. Halofuginone and amprolium were hauled out from bird tissues, and eggs using acetonitrile, followed by dehydration with the help of anhydrous sodium sulfate and the addition of hexane saturated acetonitrile. Later, the hexane layer was discarded. The acetonitrile layer was evaporated and reconstituted with the help of the mobile phase, filtered, then analyzed using LC. In addition to the method adopted to extract the drugs from eggs, the dehydrated acetonitrile solution was evaporated to 5 ml, followed by SPE, eluted by 15ml of acetonitrile+water (95+5), evaporated, reconstituted by mobile phase, filtered then subjected to analysis using LC-DAD. The chromatographic separation was undertaken on TSK-gel ODS-80TM column using the mobile phase constituted of McIlvaine buffer (pH 3.4): acetonitrile, containing 0.01M sodium lauryl sulfate. UV detection of halofuginone and amprolium was done at 242 and 265 nm wavelengths, respectively. The recoveries of halofuginone and amprolium from poultry tissue, fortified at 500 ng/gm, were 94.2±5.0% and 74.8±17.7, respectively (n=10). In bird tissue, the detection limit for both amprolium and halofuginone was 30 ng/g. The recovery of the amprolium and halofuginone using SPE, during the cleanup procedure was 85.0 ± 2.4% and 54.6 ± 3.4%, respectively. The limit of detection for both halofuginone and amprolium was 40 ng/g.

Furusawa (2002) developed a simple liquid chromatographic method for routine monitoring of amprolium residue in poultry tissues *viz.* muscle and liver, using HPLC-PDA, after sample cleanup. For the chromatographic separation, identification, and determination, Mightysil RP-4 GP column, along with the mobile phase, composed of ethanol-5mM: 1-heptane sulfonic acid sodium salt solution (36:65, v/v) with a PDA detector were used. The tissues were fortified at 0.3 to 3 μg/gm, and over 90% recoveries were recorded. The LOQ obtained was 220 ng/gm for muscle and 250 ng/gm for the liver.

Byung-Ju Kim *et al.*, (2012) developed a critical technique for concurrent estimation of decoquinate and amprolium in cattle and poultry muscle by HPLC-UV. Samples were subjected to extraction of amprolium and decoquinate using the HLB-SPE cartridge with methanol and acetonitrile. The samples were eluted using methanol,

evaporated under nitrogen, reconstituted with solvent (methanol: Water, 1:1). Chromatographic separation was achieved with the help of RP C_{18} column, under gradient elution with 20 Mm HFBA as A and MeOH: ACN (1:1.8) as B. The fortified blank samples were used for the standard curves (r^2 = 0.997), the recoveries of which ranged from 78 to 107%, and the achieved LOQ was 130-420 µg/kg.

CHAPTER-III

Materials and Methods

CHAPTER-III

MATERIALS AND METHODS

Objective

Detection and quantification of antibiotics (with special reference to presence of sulphaquinoxaline and amprolium) in poultry meat sold in and around Jammu region.

3.1 Technical Programme of Work

3.1.1 Experimental Design

3.1.1.1 Sample collection, handling, and preservation:

Over 100 samples of broiler chickens were collected randomly from October 2020 onwards from local markets of the Jammu region, which included twenty samples each from Kathua, Samba, Jammu, Reasi, and Udhampur for estimation of sulphaquinoxaline. Over sixty samples of chicken tissues *i.e.,* fifteen samples each from Kathua, Samba, Jammu and Reasi were gathered for estimation of amprolium as well. Each sample (Liver, kidney, and muscle) was labeled, packed suitably with dates and places of collection, and transported under an aseptic condition in an icebox to the Division of Pharmacology and Toxicology F.V.Sc and A.H., R.S. Pura Jammu for analysis. The samples were stored in deep freeze (-20°C) until analysis.

3.1.1.2 Chemicals and Solutions

In order to detect sulphaquinoxaline and amprolium the chemicals used during this study, like acetic acid, methylene chloride, ammonium acetate, and n-hexane, phosphate buffer were procured from HiMedia Laboratories, Mumbai, India. The chromatography solvents like water, methanol, acetonitrile, acetone etc., were procured from Merck.

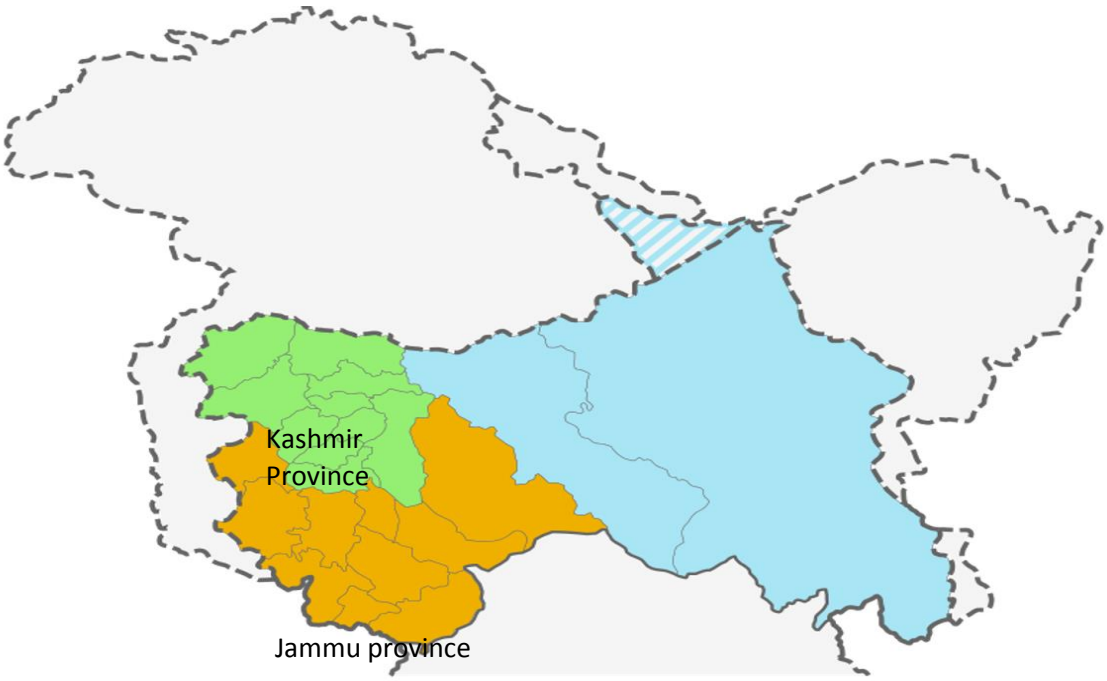


Figure 3.1: Map of Jammu and Kashmir

Figure 3.2: Map of various districts under the Jammu province

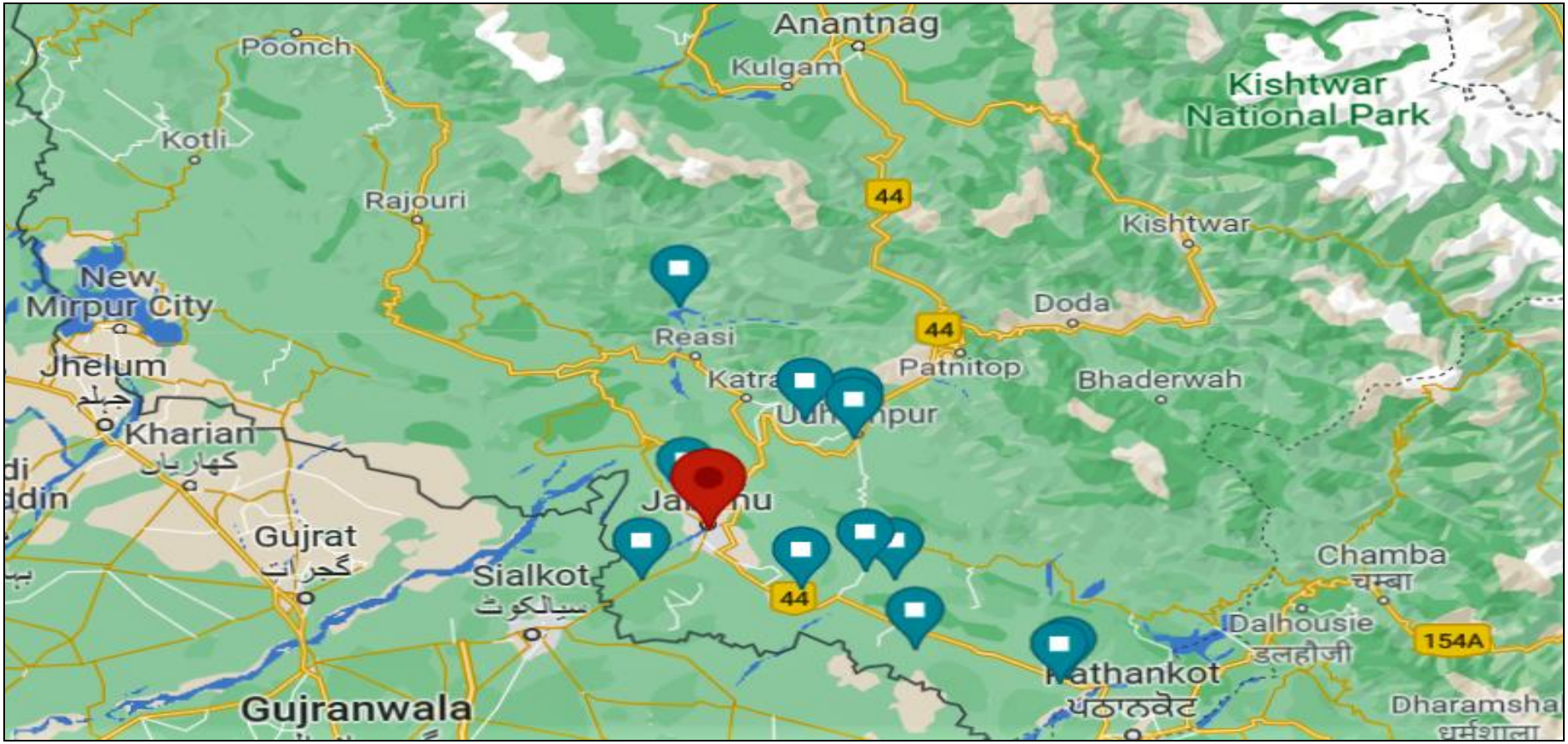


Figure 3.3: The locations (in green) of samples collection on the map of the Jammu

3.1.1.3 Equipments

The equipments used during this study include vortex mixer (Yorco sales Pvt., Ltd., India), porcelain mortar pestles, tissue homogenizer (IKA T10 Basic Ultra Turrax), ultrasonic homogenizer (Optics Technology), refrigerated centrifuge (R.H.152, UNILAB), vacuum filtration assembly (Solvkitin, Merck Life Science Pvt. Ltd., Bangalore), pH meter (Eutech Instruments pH510) and BOD incubator (Relitech, Pragat Laboratory Equipments, Ambala, India) were used for sample preparation.

3.1.1.4 Instrumentation and chromatographic conditions

The analysis of sample was accomplished using Shimadzu *Prominence i* 2030C plus HPLC system, equipped with quaternary pump, degasser, auto sampler, UV-VIS and RF 20A detector along with inbuilt "LABSOLUTION" software. The reverse-phase chromatography was performed using Purospher® Star RP18 end capped (5µ) HPLC column (250 mm-4.6). The mobile phase was sieved using a 0.45 µm nylon filter (Millipore) followed by sonication for minutes. The detection was performed using a UV/VIS detector set at 266 nm. The method was optimized using binary-gradient mobile phase with HPLC grade water containing 0.01M ammonium acetate (pH 4.6) as mobile phase A and acetonitrile (ACN) as B, in the ratio of 43:57, for 7 minutes at 1ml/minute flow rate, and the injection volume used for analysis was 20 µl (Cheong *et al*., 2010). The chromatographic separation for amprolium was achieved by isocratic mode with 0.05 M phosphate buffer (pH 5) and acetonitrile (30:70; v/v) as mobile phase and the detection was performed using UV/VIS detector set at 263 nm (Mantri *et al*., 2015).

3.1.1.5 Preparation of standard solutions

The stock solution of sulphaquinoxaline (SQX=1mg/ml) was prepared by dissolving 0.01g of SQX standard with 10ml of 90% acetonitrile (n-hexane saturated). This stock solution was further diluted progressively to prepare working standard solutions (8 to 1 and 0.5, to 0.125 µgm/ml). The working standard solutions for

calibration, were prepared by diluting stock solutions with 50% methanol in 0.01M ammonium acetate (pH 4.6).

The stock solution of amprolium (1000 µg/ml) was prepared by dissolving 10 mg of amprolium standard in mobile phase. Further, progressive dilutions were made from this stock solution in mobile phase in the concentrations range of 10 to 0.015625 µg/ml.

3.1.1.6 Extraction technique and cleanup procedure for analysis of sulphaquinoxaline

Extraction of sulphaquinoxaline from tissue samples (liver, muscle and kidney) was carried out as per the method of Cheong *et al.* (2010) with suitable modifications. The collected frozen tissues were left to thaw, then cut to pieces, weighed (4gm), and triturated well, using mortar pestles or tissue homogenizer (IKA T 10 Basic Ultra Turrax). Then, 12 ml 90% acetonitrile (n-hexane saturated) was added to the triturated tissue in mortar and pestle, followed by homogenization and centrifuged at 4000 rpm for 10 minutes. The supernatant was then transferred into a round bottom flask (as A), whereas the residue was extracted again with 8ml acetone followed by sonication for 5 minutes, centrifuged at 4000 rpm for 10 minutes, the supernatant of which conferred as B. Later, both supernatants A and B were mixed and poured in another pear-shaped flask, and evaporated at 50°C in BOD until near to dryness. Later, 6 ml methylene chloride was added, mixed using vortex mixer, transferred into petri discs and allowed to dry at 50°C. The solution was reconstituted with 1ml 50% methanol in acetate buffer and mixed, 2ml n-hexane was added, mixed using vortex mixer, left to stand for few minutes followed by liquid: liquid extraction in falcon tube. The n-hexane layer was discarded and the left-over mix of ammonium acetate buffer and methanol was obtained, filtered using syringe filter (0.22µ, Millipore, Millex-GV), and the filtrate was placed in auto sampler rack. The HPLC system was then programmed for 20 µl injection for analysis. The flow diagram of the procedure is represented in figure 3.4.

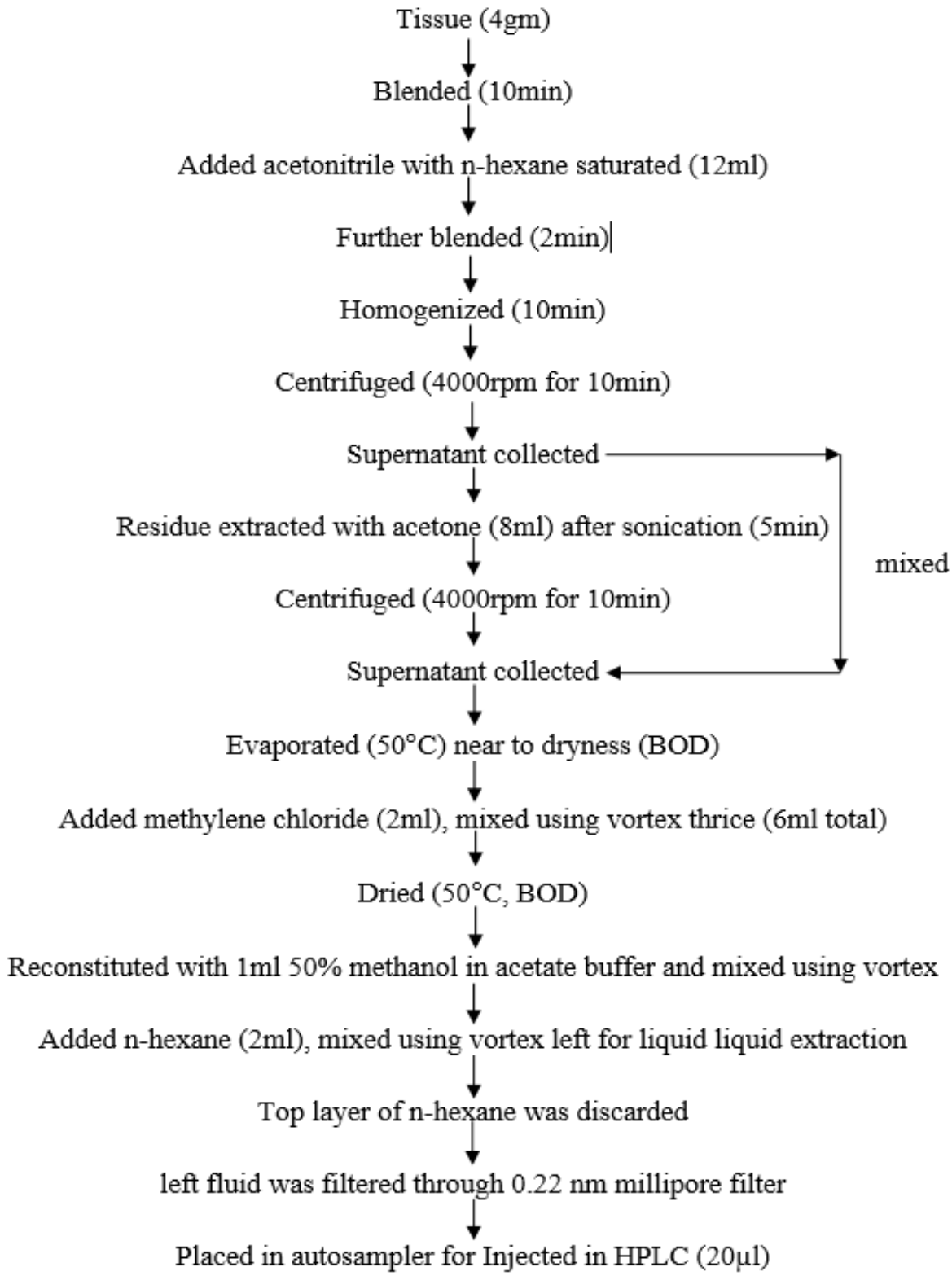


Figure 3.4: Flow diagram of sample clean-up for the analysis of sulphaquinoxaline

3.1.1.7 Extraction techniques and clean-up procedure for analysis of amprolium

Extraction of amprolium from tissue samples (liver, muscle and kidney) was carried out as per the method of Byung-Ju Kim *et al.* (2011) with little suitable modifications. The samples were taken out of deep freeze (-20℃) to thaw, then 4g of tissue was cut into small pieces, triturated in mortar with the help of pestle followed by addition of acetonitrile (10 ml), again homogenized and sonicated for 10 minutes. The homogenized sample was transferred in 50 ml falcon tube, centrifuged for 15 minutes at 4000 rpm. The supernatant was then mixed with 1mL of HPLC grade water. The mixture solvent was vaporized down to 1mL at 60°C (BOD) followed by addition of 4 ml HPLC grade water. An SUPEL SELECT HLB SPE cartridge was connected to vacuum manifold and conditioned by a solution of 4 ml acetonitrile and 4 ml distilled water then 5 mL of sample was loaded into the cartridge. The sample was eluted with 5 mL acetonitrile twice and the eluent was completely vaporized at 60°C (BOD). The residue was dissolved with 2 ml of reconstitution solvent (phosphate buffer (0.05M, pH 5): acetonitrile in 1:1 ratio) and then filtered using 0.22 µm syringe filter. The filtrate (1.5 ml) was placed in auto-sampler rack for injection (20 µl) in HPLC system for analysis. The procedure is shown in figure 3.5.

3.1.1.8 Validation of the high-performance liquid chromatography method

The HPLC assay was validated by determining linearity, selectivity, accuracy, range, precision, Limit of Detection (LOD) and Limit of Quantification (LOQ), robustness, ruggedness, noise, sensitivity.

Selectivity

Selectivity of an analytical method is its ability to measure an analyte of interest precisely in the presence of interferences due to presence of sample matrix therein. It is cross checked by inspecting chromatographic blanks (from a sample without the analyte) in the expected time window of the analyte peak.

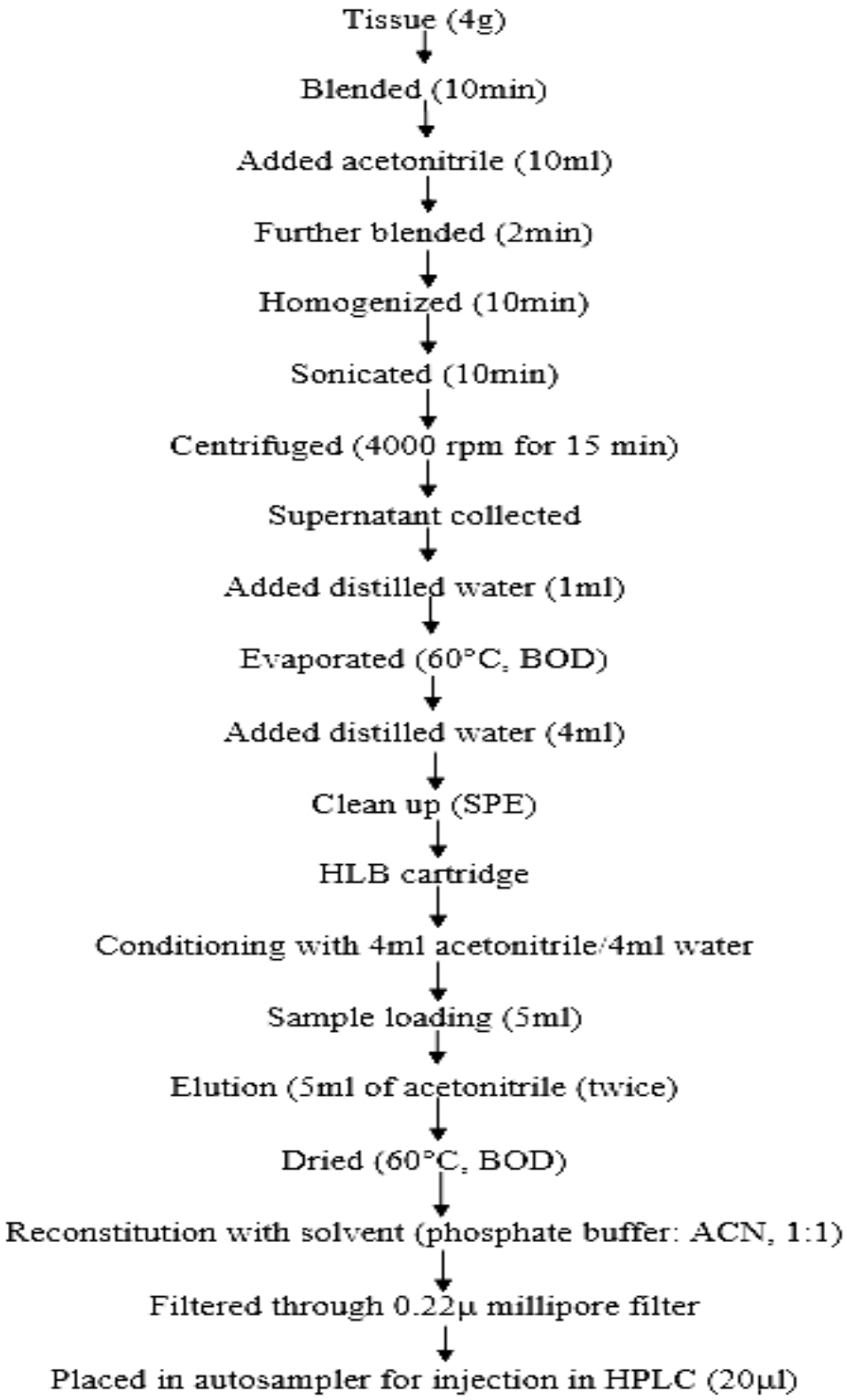


Figure 3.5: Flow diagram of sample cleanup for the analysis of amprolium

Linearity

The linearity of the analytical procedure is its ability to obtain resultant chromatograph (in HPLC) that are directly proportional to the concentration of analyte of interest in the sample within the given range, or it may be proportional by means of well-defined mathematical transformation. Data from the calibration curve provides an estimation of the degree of linearity.

Detection limit and quantification limit

The term LOD refers as the least concentration at which the instrument can detect, and the noise to signal ratio for detection limit should be 1:3. The term quantification limit refers to the least concentration of the analyte at which the analytical equipment can detect and quantify. The noise to signal ratio for quantification limit should be 1:10. Detection limit and quantification limit are calculated by considering the noise to signal ratio of the least concentration of linearity samples and is expressed in µg/ml or ppm. The sensitivity of the method of analysis can be observed from the value of the detection limit and the limit of quantification. A method with better sensitivity will have a lower value of LOD and LOQ.

Calculation of LOD and LOQ values for instrument sensitivity

LOD (mg/ml) = 3×Noise/signal×lowest concentration of the linearity samples

LOQ (mg/ml) = 10× Noise/signal×lowest concentration of the linearity samples

Accuracy and precision

Accuracy is the closeness of an individual test result to the true value. It is expressed as percent recovery by the assay of a known amount of analyte added. It is calculated from test results as the percentage of the analyte recovered by the assay. Precision is characterized as measure of the degree of reproducibility/ repeatability of the analytical method under normal operating circumstances.

Range

The range of an analytical technique is the interlude in between the highest and lowest concentration of analyte in the sample for which it has been confirmed that the

analytical technique has an appropriate and suitable level of precision, accuracy and linearity.

Robustness

It is the capacity of the analytical method to continue and remain unaltered by small but deliberate change in method parameters and delivers an indication of its dependability during normal usage.

Ruggedness

It is the extent of reproducibility of analytical tests' results achieved by the scrutiny of the similar sample under a variation of normal test conditions within the specified parameters of the assay. The repeatability can be equated to the precision and exactness of the assay under standard condition to obtain the degree of the ruggedness of the analytical method.

Noise

It refers to rapid changes in the strength and regularity of a recorded sign irrespective of the existence or absence of the analyte. It is the difference between the highest and lowest value of the measured signal without the presence of analyte, observed in a relatively short span, as compared to the time-span necessary for measurement of the analyte.

Sensitivity

The change of measured signal as a result of one unit change in the content of an analyte. The change is calculated from the slope of the calibration line of the analyte.

Calculation of percent recovery

The accuracy of an analytical method is usually determined by the recovery (%) of the drug quantitatively added to blank biological specimens before sample preparation and assay following the corresponding procedure. A high and constant recovery is particularly important and relevant for a standard HPLC analysis. The percent recovery

was calculated by taking means of peak area of each concentration of drug standard in plasma as well as tissues in triplicate. Recovery of sulphaquinoxaline residues was calculated by employing the following formula.

$$Recovery\ \% = \frac{N(\sum xy) - (\sum x)(\sum y)}{N(\sum x^2) - (\sum x)^2} \times 100$$

Where x = amount of standard drug added, y = amount of drug found by proposed method, n = number of observation.

Calculation of correction factor

Correction factor (c.f.) for a particular residue factors was calculated by the following formula for each of residual concentrations (Leoni *et al*., 1992).

C.f. = 100/ Percent recovery

CHAPTER-IV

Results

CHAPTER-IV

RESULTS

4.1 Sulphaquinoxaline: Limit of detection and limit of quantitation

The extent of detection limit (LOD) is considered to be the concentration of the concerned analyte (sulphaquinoxaline) which leads to a clear detector response, equals to 3:1 ratio, approximately to the background noise (Desimoni and Brunetti, 2015). The quantitation limit (LOQ) is the lowest amount that can be analyzed within acceptable precision and accuracy at signal to noise ratio of 10:1 (Biswas *et al.*, 2007). LOD and LOQ were estimated following running of various dilution of standard in the range of 0.00390625 to 8 µgm ml^{-1} in triplicate in UV-HPLC and the peak of retention time (Rt) for standard sulphaquinoxaline on an average was 3.813 minutes. The area under curve for different concentration of sulphaquinoxaline were recorded (table 4.1) for calculation of LOD and LOQ using MS Excel software and the table 4.2 and 4.3 represents regression analysis. The calibration curve and value of r^2: 0.9999 was calculated using "LABSOLUTION" software inbuilt with HPLC system illustrated in figure 4.1. LOD and LOQ for sulphaquinoxaline were quantified as 15.427 ng/ml and 46.749 ng/ml.

Table 4.1: Estimation of LOD and LOQ of sulphaquinoxaline

S. No.	Concentration of sulphaquinoxaline µg/ml	Area under curve (AUC)
i.	8	819579
ii.	8	821491
iii.	8	819247
iv.	4	408121
v.	4	407382
vi.	4	407892
vii.	1	109402
viii.	1	108312

ix.	1	109634
x.	0.5	55505
xi.	0.5	49196
xii.	0.5	52350
xiii.	0.25	24409
xiv.	0.25	24253
xv.	0.25	24253
xvi.	0.125	16659
xvii.	0.125	12464
xviii.	0.125	12465
xix.	0.0625	6427
xx.	0.0625	6533
xxi.	0.0625	6530
xxii.	0.03125	3563
xxiii.	0.03125	3606
xxiv.	0.03125	5983
xxv.	0.015625	1706
xxvi.	0.015625	1658
xxvii.	0.015625	1864
xxviii.	0.0078125	1134
xxix.	0.0078125	1066
xxx.	0.00390625	585
xxxi.	0.00390625	635
xxxii.	0.00390625	686

Table 4.2: Regression analysis for estimation of LOD and LOQ of sulphaquinoxaline

ANOVA					
	Df	***SS***	***MS***	***F***	***Significance F***
Regression	1	1.99591E+12	1.99591E+12	338309.2	3.66354E-64
Residual	31	182889268.4	5899653.819		
Total	32	1.99609E+12			

Table 4.3: Regression analysis for estimation of LOD and LOQ of sulphaquinoxaline

	Coefficients	***Standard Error***	***t Stat***	***P-value***	***Lower 95%***	***Upper 95%***
Intercept	873.858	478.429	1.826	0.077	-101.904	1849.621
X Variable1	102337.999	175.946	581.643	3.66E-64	101979.153	102696.843

$$LOQ = \frac{10 \times Standard\ Error\ intercept}{Coefficient\ of\ variable} = \frac{10X478.4294756}{102337.999}$$

$$= 0.04674\ \mu g/gm\ or\ 46.749\ ng/gm$$

$$LOD = \frac{3.3 \times Standard\ Error\ intercept}{Coefficient\ of\ variable} = \frac{3.3X478.4294756}{102337.999}$$

$$= 0.015427\ \mu g/gm\ or\ 15.427\ ng/gm$$

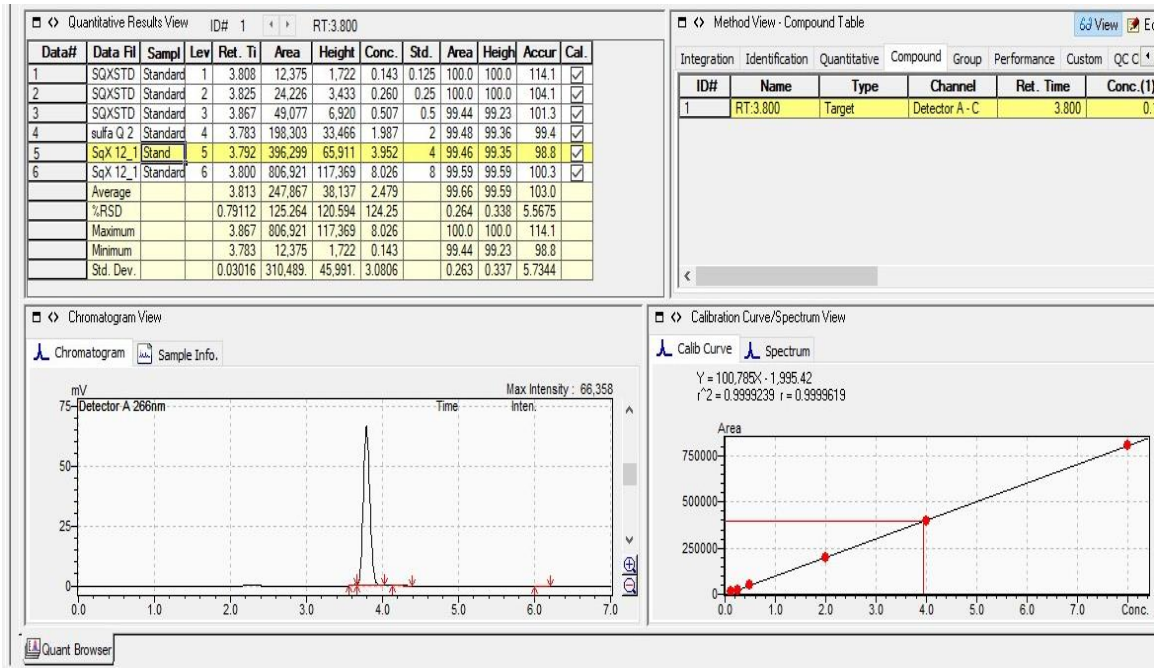
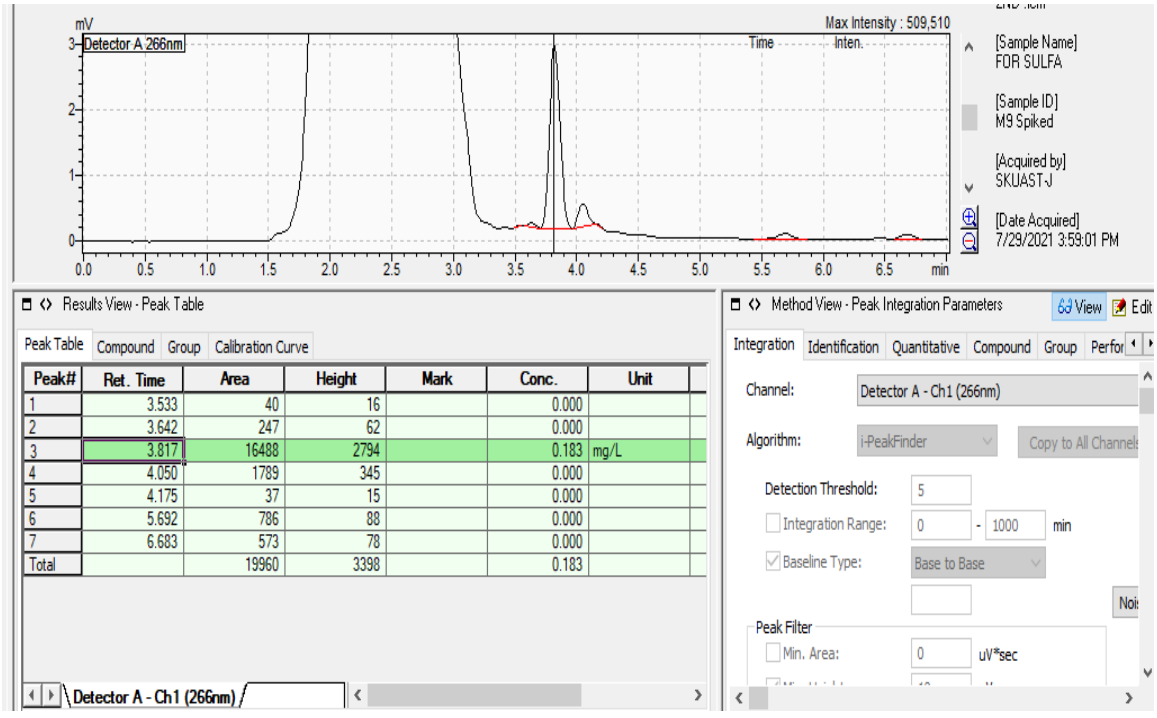


Figure 4.1: Calibration curve of sulphaquinoxaline using LABSOLUTION software



Peak#	Ret. Time	Area	Height	Mark	Conc.	Unit
1	3.533	40	16		0.000	
2	3.642	247	62		0.000	
3	3.817	16488	2794		0.183	mg/L
4	4.050	1789	345		0.000	
5	4.175	37	15		0.000	
6	5.692	786	88		0.000	
7	6.683	573	78		0.000	
Total		19960	3398		0.183	

Figure 4.2: Chromatogram showing the presence of sulphaquinoxaline residue in unknown chicken tissue sample

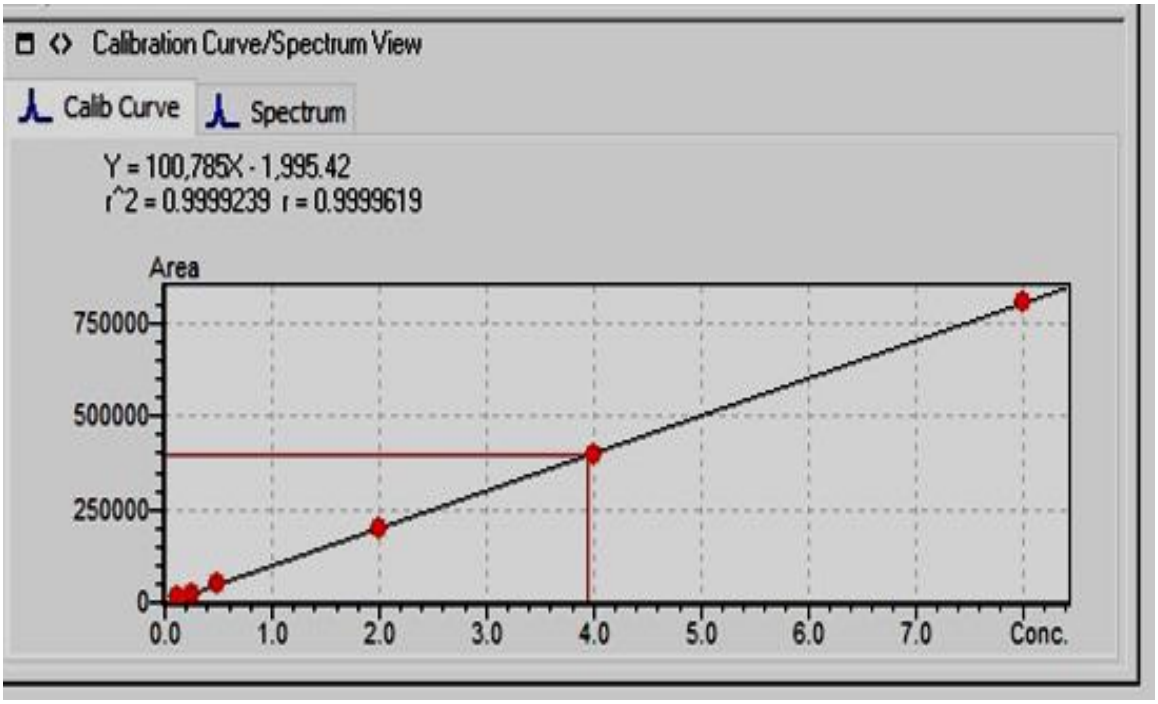


Figure 4.3: Standard curve (zoom view) as depicted under HPLC operating LABSOLUTION software

4.1.1 Recovery of sulphaquinoxaline

The samples were spiked using sulphquinoxaline @ 0.3 µg/gm in each of kidney, liver and muscle, in triplicate and were analyzed for recovery and presented in the table 4.4.

Table 4.4: Estimation of recovery percentage of sulphaquinoxaline in chicken tissue

Organs	Recovery percentage ± SD (%)
Kidney	78.900 ±0.134
Liver	77.484 ± 0.313
Muscle	86.838 ± 0.050

4.1.2 Analysis of collected tissue samples for sulphaquinoxaline residue

Sulphaquinoxaline detection and quantification was accomplished using the method reported by Cheong *et al,* (2010), with suitable modification, and standard calibration curve was obtained using the "LABSOLUTION" software (figure 4.1). The chicken samples (n=20) (liver, kidney and muscles) were collected from five districts of Jammu province *i.e.*, Jammu, Kathua, Samba, Reasi and Udhampur. The samples were subjected to analysis following sample preparation and cleanup to injection in HPLC system.

The liver, kidney and muscles tissues of chicken sold in district Jammu has been analyzed for the presence of sulphaquinoxaline residue and the data has been presented in the table 4.5. It can be observed (table 4.5) that all the samples of liver and kidney, and majority (90%) of muscles collected from the sampling region of Jammu district were positive for the presence of sulphaquinoxaline residue. However, 70 percent of the samples of liver, 10 % of kidney and 25% of muscles samples gathered from district Jammu, were found to have higher residue than established international MRL (EC, 2010). The minimum level of sulphaquinoxaline residue found was 26.869 and 6.401µg/kg in liver and kidney, respectively, though it remained undetected in the muscle tissues, collected from Jammu district. The highest concentration of sulphaquinoxaline found in liver, kidney and muscles were 814.553, 490.862 and 315.567µg/kg in liver, kidney and muscle tissues, respectively.

The table 4.6 exhibits that 75% of kidney and 80% of muscle tissues gathered from Udhampur were positive for sulphaquinoxaline, and none of the kidney and muscles tissues exhibited residues over MRL. Although, 100% of the gathered liver exhibited residue of sulphaquinoxaline beyond established MRL of 100 µg/kg (EC, 2010). It can be vouched from the table that the minimum residue concentration of sulphaquinoxaline found in liver was 138.04 µg/kg, although the same remained undetected in kidney and muscle tissues. The maximum concentration of tissue sulphonamide residue in liver, kidney and muscle were 964.76, 44.087 and 51.409 µg/kg in liver, kidney and muscles, respectively.

The data presented in table 4.7 shows the status of sulphaquinoxaline residue in tissues of collected from Samba that the samples of liver, kidney and muscles were positive

to the extent of 90%, 85% and 55%, respectively, although, 50% of the liver tissues crossed the prescribed MRLs, other tissues (kidney and muscles) had residues well below established MRL values (EC, 2010). The minimum level of tissues sulfonamide residues remained undetected in the samples collected from district Samba. However, the maximum level of sulphaquinoxaline residues in these samples were 682.03, 55.132 and 39.475 µg/kg in liver, kidney and muscles, respectively.

The residue status for sulphaquinoxaline in samples gathered from Reasi are presented in table 4.8. The samples of liver, kidney and muscles were positive to the extent of 85%, 65% and 70% of respectively, though only 25% of the liver tissues had sulphaqinoxaline residue, over the established MRL (EC, 2010). Few chicken samples from Reasi, of liver, kidney and muscles under study were negative for the sulphaquinoxaline residues, though the highest values of the residues was 125.342, 4.209 and 8.917 µg/kg, respectively.

The table 4.9 depicts the residue status of sulphaquinoxaline in the samples collected from Kathua, Jammu. Majority of the collected samples from Kathua were positive for sulphaquinoxaline residue, to the extent of 100% of liver, 80% of kidney and 85% of the muscle, however, only liver samples to the extent of 45% had residue beyond the established MRL (EC, 2010). Some of the chicken samples from Kathua, Jammu under study were negative, though the liver tissue exhibited minimum of 14.265 µg/kg for the sulphaquinoxaline residues. However, the maximum values for sulphaquinoxaline residues quantified was 549.734, 25.342 and 40.159 µg/kg in liver, kidney and muscle tissues, respectively.

Table 4.5: The residue of sulphaquinoxaline (μg/kg) in chicken tissues samples (n=20) collected from vendors of district Jammu, UT of J&K.

Maximum residue level, sulphaquinoxaline			
EC, 2010	**100 μg/kg**	**100 μg/kg**	**100 μg/kg**
S. No.	**Liver**	**Kidney**	**Muscle**
1.	655.917	29.261	16.544
2.	631.800	44.316	101.205
3.	26.869	28.583	4.214
4.	562.126	40.559	56.093
5.	140.001	12.973	4.214
6.	193.390	8.385	20.436
7.	516.281	13.869	0.326
8.	814.553	12.973	20.436
9.	95.507	15.123	11.486
10.	67.229	490.862	4.110
11.	100.530	218.253	44.645
12.	123.638	23.110	83.521
13.	50.936	35.341	245.206
14.	298.714	40.559	42.979
15.	40.252	18.194	29.188
16.	439.687	7.580	12.621
17.	52.142	26.293	135.147
18.	143.041	9.583	ND
19.	212.938	15.582	315.5677
20.	240.763	6.401	275.1003
Mean	270.316	54.890	71.152
Minimum residue concentration	26.869	6.401	ND
Maximum residue concentration	814.553	490.862	315.567
Standard error	54.387	25.095	21.698
Positive (%)	100	100	90
≥MRL	14	2	5
≥MRL (%)	70	10	25

Table 4.6: The residue of sulphaquinoxaline (μg/kg) in chicken tissues samples (n=20) collected from vendors of district Udhampur, Jammu, J&K (UT).

Maximum residue level, sulphaquinoxaline			
EC, 2010	**100 μg/kg**	**100 μg/kg**	**100 μg/kg**
S. No.	**Liver**	**Kidney**	**Muscle**
1.	233.2173	ND	12.04223
2.	292.3575	ND	ND
3.	139.0606	7.57909	3.538367
4.	652.1524	16.39913	7.249691
5.	178.048	5.389837	15.66189
6.	210.5617	0.784481	11.34808
7.	194.1012	ND	ND
8.	138.0407	5.460196	ND
9.	665.9174	16.56053	7.696569
10.	231.011	2.803789	14.27176
11.	618.6715	3.214815	5.420695
12.	504.5431	24.80585	18.76621
13.	693.0148	ND	4.006045
14.	547.0625	19.543035	33.90403
15.	631.0707	14.390713	28.26123
16.	867.1175	33.80743	40.21735
17.	545.7113	44.08764	27.75149
18.	145.5743	29.87531	51.40931
19.	964.7603	ND	44.30157
20.	149.312	26.06734	9.35621
Mean	430.065	12.538	16.759
Minimum residue concentration	138.04	ND	ND
Maximum residue concentration	964.76	44.087	51.409
Standard error	59.693	2.990	3.500
Positive (%)	100	75	80
≥MRL	20	0	0
≥MRL (%)	100	0	0

Table 4.7: The residue of sulphaquinoxaline (μg/kg) in chicken tissues samples (n=20) collected from vendors of district Samba, Jammu J&K (UT).

Maximum residue level, sulphaquinoxaline			
EC, 2010	**100 μg/kg**	**100 μg/kg**	**100 μg/kg**
S. No.	**Liver**	**Kidney**	**Muscle**
1.	516.9786	9.579253	0.614025
2.	48.64225	ND	ND
3.	ND	89.92265	ND
4.	128.7777	3.514782	ND
5.	28.84067	0.894878	ND
6.	37.72071	0.513971	0.548871
7.	91.8241	4.329148	ND
8.	93.8123	ND	ND
9.	43.25668	ND	ND
10.	489.4699	6.982252	0.614025
11.	413.1108	7.078296	39.47594
12.	534.4308	44.43638	0.769845
13.	47.29659	2.062026	0.575536
14.	163.4374	5.774866	1.585469
15.	316.5338	4.673925	ND
16.	99.42609	0.792736	ND
17.	682.0304	6.817095	0.705671
18.	44.92104	7.074853	3.518347
19.	460.3734	55.13267	6.783127
20.	140.3065	25.09632	16.03257
Mean	219.059	13.733	3.560
Minimum residue concentration	ND	ND	ND
Maximum residue concentration	682.03	55.132	39.475
Standard error	48.164	5.216	2.068
Positive (%)	90	85	55
≥MRL	10	0	0
≥MRL (%)	50	0	0

Table 4.8: The residue of sulphaquinoxaline (μg/kg) in chicken tissue samples (n=20) collected from vendors of Reasi, Jammu, J&K (UT).

Maximum residue level, sulphaquinoxaline			
EC, 2010	**100 μg/kg**	**100 μg/kg**	**100 μg/kg**
S. No.	**Liver**	**Kidney**	**Muscle**
1.	9.971284	ND	ND
2.	14.85291	2.128503	1.595023
3.	ND	ND	0.442296
4.	23.07917	ND	ND
5.	22.86724	2.221765	2.547062
6.	8.159222	ND	ND
7.	9.595492	4.706321	0.740672
8.	ND	ND	ND
9.	24.0413	ND	ND
10.	ND	1.116071	ND
11.	118.4737	1.414188	1.650116
12.	7.393963	0.251797	4.594049
13.	123.7543	2.708515	8.917841
14.	11.08586	0.7432132	2.181165
15.	125.3421	4.618391	0.527486
16.	39.15517	1.734231	2.334487
17.	29.21575	ND	1.265431
18.	108.0554	4.239181	1.388284
19.	55.36512	3.224198	2.654327
20.	111.4321	5.209671	3.021645
Mean	42.091	1.715	1.692
Minimum residue concentration	ND	ND	ND
Maximum residue concentration	125.342	5.209	8.917
Standard error	10.446	0.410	0.476
Positive (%)	85	65	70
≥MRL	5	ND	ND
≥MRL (%)	25	ND	ND

Table 4.9: The residue of sulphaquinoxaline (μg/kg) in chicken tissues samples (n=20) collected from vendors of Kathua, Jammu, J&K (UT).

Maximum residue level, sulphaquinoxaline			
EC, 2010	**100 μg/kg**	**100 μg/kg**	**100 μg/kg**
S. No.	**Liver**	**Kidney**	**Muscle**
1.	94.25247	11.84432	14.38839
2.	51.92639	18.52905	26.27652
3.	549.7344	25.34205	40.15912
4.	47.47821	15.06214	9.629948
5.	412.1104	7.013219	17.75355
6.	100.30951	ND	1.943066
7.	32.33204	0.851752	ND
8.	19.37731	ND	ND
9.	17.85641	0.461421	0.325910
10.	120.2544	11.5548	23.08352
11.	23.36945	3.214534	0.762125
12.	510.3735	22.52304	44.31624
13.	87.32256	ND	0.32191
14.	14.26519	ND	ND
15.	318.0678	0.416371	0.850664
16.	57.54723	1.236041	5.206175
17.	116.0365	1.14557	1.226049
18.	612.1206	5.772197	40.14362
19.	67.9123	3.434457	4.259653
20.	130.3457	4.756406	19.52208
Mean	169.149	6.657	12.50805
Minimum residue concentration	14.265	ND	ND
Maximum residue concentration	549.734	25.342	40.159
Standard error	43.619	1.805	3.376
Positive (%)	100	80	85
≥MRL	9	0	0
≥MRL (%)	45	0	0

4.2 Amprolium: Limit of detection (LOD) and limit of quantitation (LOQ)

The area under curve for different concentrations of amprolium was recorded and presented in table 4.10. The calibration curve (figure 4.4) and value of r^2: 0.9998 was calculated using inbuilt HPLC operating software "LABSOLUTION" (figure 4.5). LOD and LOQ were estimated using MS excel software and the regression analysis is presented in table 4.11 and 4.12, following running of various dilutions of standard in the range of 20 to 0.00781 µg/ml in triplicate in UV-HPLC. The peak of retention time (average) for standard amprolium was found 2.663 minutes. LOD and LOQ for amprolium was estimated as 145 ng/ml and 441 ng/ml, respectively.

Table 4.10: Estimation of LOD and LOQ for amprolium

S.No.	Concentration of amprolium (µg/ml)	Area under curve (AUC)
i	20	702169
ii	20	704586
iii	20	728996
iv	10	338356
v	10	324508
vi	10	323352
vii	5	167609
viii	5	162254
ix	5	161676
x	2.5	80786
xi	2.5	81127
xii	2.5	80838
xiii	1.25	39900
xiv	1.25	39837
xv	1.25	39798
xvi	0.5	16359

xvii	0.5	16507
xviii	0.5	16588
xix	0.625	19551
xx	0.625	19377
xxi	0.625	19354
xxii	0.3125	9547
xxiii	0.3125	9548
xxiv	0.3125	9507
xxv	0.125	3846
xxvi	0.125	3916
xxvii	0.125	3893
xxviii	0.0625	1977
xxix	0.0625	2183
xxx	0.0625	2559
xxxi	0.03125	1565
xxxii	0.03125	1542
xxxiii	0.03125	1549
xxxiv	0.015625	934
xxxv	0.015625	892
xxxvi	0.015625	900
xxxvii	0.00781	378
xxxviii	0.00781	367
xxxix	0.00781	390

Table 4.11: Regression analysis for estimation of LOD and LOQ of amprolium

ANOVA					
	df	***SS***	***MS***	***F***	***Significance F***
Regression	1	1.5106E+12	1.5E+12	21093.7	1.3E-52
Residual	37	264970831	7.2E+07		
Total	38	1.5133E+12			

Table 4.12: Regression analysis for estimation of LOD and LOQ of amprolium

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	-3148.1	1549.956	-2.0311	0.04947	-6288.6	-7.628
X Variable1	35137.5	241.932	145.237	1.3E-52	34647.3	35627.7

$$LOQ = \frac{10 \times Standard\ Error\ intercept}{Coefficient\ of\ variable} = \frac{10 \times 1549.95621}{35137.5}$$
$$= 0.44111\ \mu g\ /\ gm\ or\ 441.111\ ng/gm$$

$$LOD = \frac{3.3 \times Standard\ Error\ intercept}{Coefficient\ of\ variable} = \frac{3.3 \times 1549.95621}{35137.5}$$
$$= 0.14556\ \mu g/gm\ or\ 145.56\ ng/gm$$

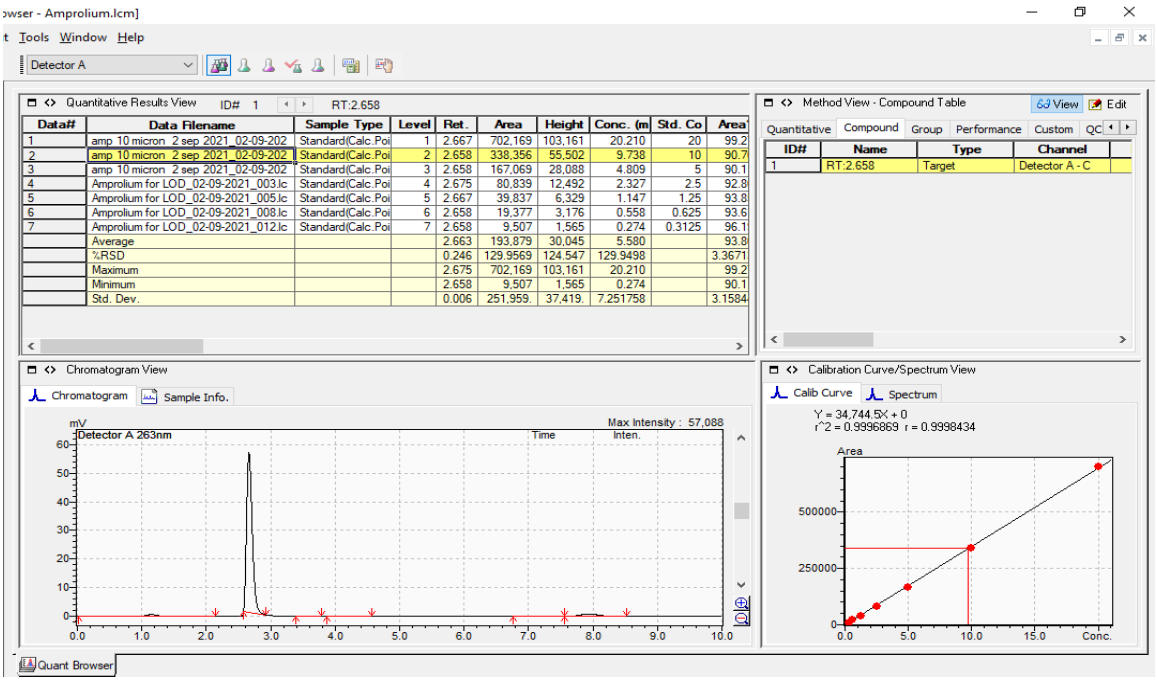


Figure 4.4: Calibration curve of amprolium using LABSOLUTION software

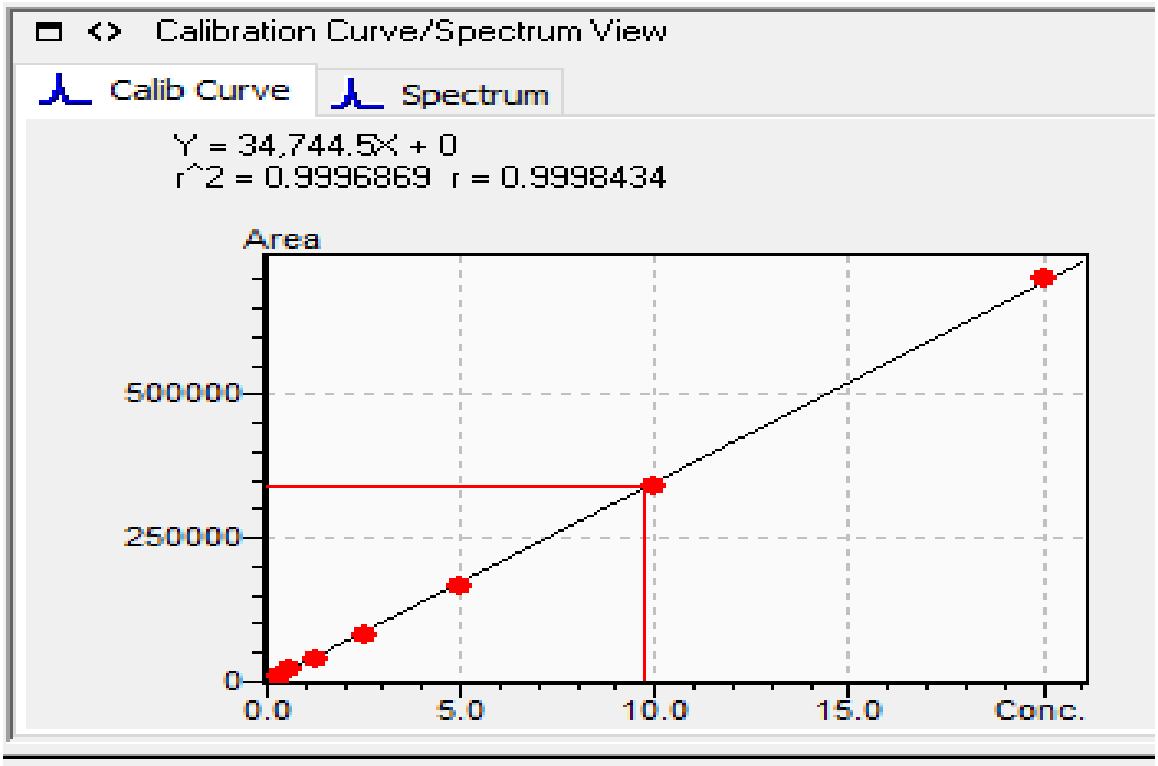


Figure 4.5: Standard curve of amprolium using LABSOLUTION software

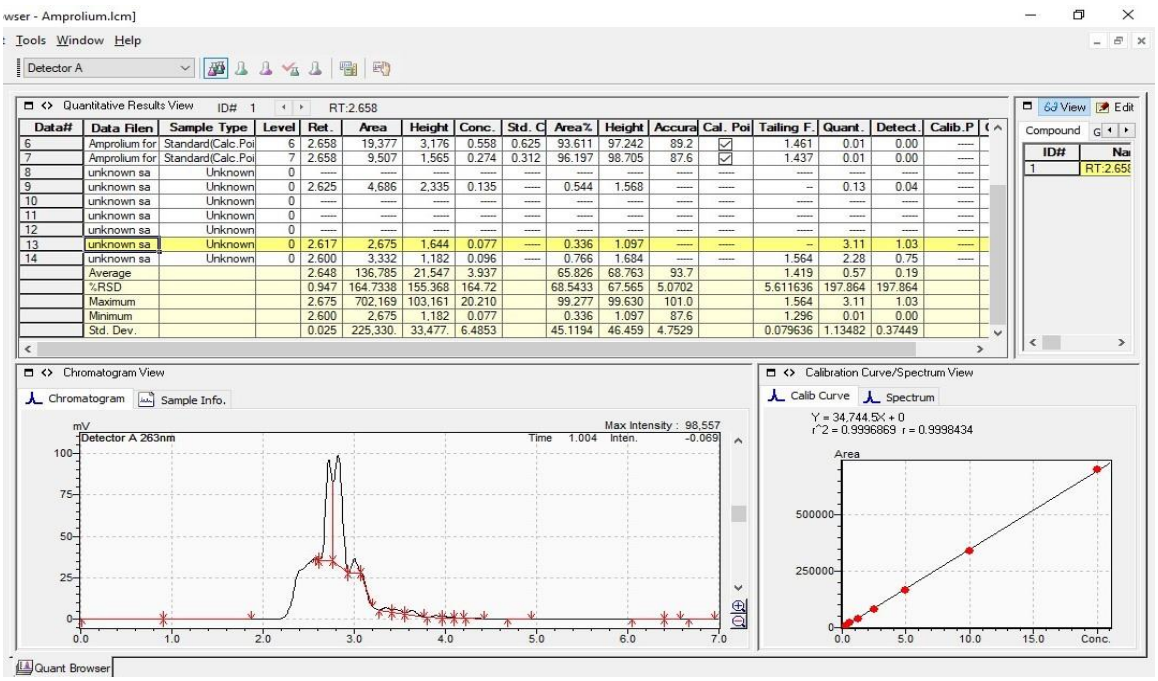


Figure 4.6: Chromatogram showing the presence of amprolium residue in unknown tissue sample

4.2.1 Recovery of amprolium

The samples were spiked using amprolium @ 400ng/gm in each of liver, kidney and muscle in triplicate and were analyzed for recovery and presented in the table 4.13. It can be vouched from the table 4.13 that the recovery percentage for kidney was approximately 68.3 percent, and was least among the organ collected for study, followed by muscles. The residue status of liver was approximately 72 percent.

Table 4.13: Estimation of recovery percentage of amprolium in chicken tissue

Organs	Recovery percentage ± SD (%)
Liver (Chicken)	72.156± 0.704
Kidney (Chicken)	68.316± 1.495
Muscle (Chicken)	71.029±0.575

4.2.2 Analysis of collected tissue samples for amprolium residue

The method for analysis of amprolium was accomplished by method reported by Mantri *et al*, (2015) and Byung-Ju Kim *et al*, (2012), with suitable modification as mentioned in materials and methods, and standard calibration curve was obtained using the LABSOLUTION software (figure: 4.4). The chicken samples (n=15) (liver, kidney and muscles) were collected from four districts of Jammu region *i.e.*, Jammu, Kathua, Samba and Reasi. The samples were subjected to analysis following sample preparation and cleanup to the placement in autosampler rack in HPLC system commanded for 20µl injection.

The established MRLs (CFR, 2020) for liver and kidney is 1000 µg/kg, and 500 µg/kg for muscle. The liver, kidney and muscles tissues of chicken sold in Jammu district, were subjected to analysis for the presence of amprolium residue, and the data has been presented in the table 4.14. It can be vouched from the table that 66.66% of the samples of liver, 73.33% of kidney, and 86.66% of muscles collected from the sampling region of Jammu district were positive for residue of amprolium.

The table 4.15 represents the tissue residues status for the samples gathered from Kathua, Jammu. The tissues were also positive for amprolium residues to the extent of 93.33%, 80% and 100% of liver, kidney and muscles respectively. Similar to the earlier findings, none of the tissues exhibited values for amprolium residues beyond established MRL (CFR, 2020). It can be vouched from the results, that none of the tissues samples collected from all four districts of Jammu were beyond established MRL for amprolium.

The table 4.16 exhibits the residue profile of tissues collected from Reasi, Jammu. It can be viewed from the table 4.16 that the tissues of liver, kidney and muscles collected from Reasi, Jammu were found positive to the extent of 86.66%, 73.33% and 86.66% respectively. However, none of the collected tissues exhibited values of residues beyond prescribed MRL (CFR, 2020).

The table 4.17 shows the tissue residue status of amprolium in Samba district of Jammu. It was found that 86.66%, 66.66 %, and 80% of the gathered samples of liver, kidney and muscles samples from district Samba, were positive for the residue of

amprolium, however, none of the samples were found beyond the established value of MRL (CFR, 2020).

Table 4.14: The residue of amprolium (µg/kg) in chicken samples (n=15) collected from vendors of district Jammu, J&K (UT)

Maximum residue level, amprolium			
FAO/WHO	**1000 µg/gm**	**500 µg/gm**	**1000 µg/gm**
S. No.	**Liver**	**Muscle**	**Kidney**
1	244.282	191.786	184.442
2	277.856	236.121	0.000
3	ND	197.770	315.380
4	226.404	221.839	232.004
5	334.626	416.752	449.285
6	479.383	305.417	386.510
7	ND	245.466	264.100
8	ND	292.749	246.529
9	104.618	281.090	ND
10	ND	ND	ND
11	167.173	143.012	137.376
12	18.191	295.817	492.587
13	128.906	ND	ND
14	297.333	151.547	352.933
15	562.614	256.745	309.380
Mean	189.426	215.741	224.702
Minimum residue concentration	ND	ND	ND
Maximum residue concentration	562.614	256.745	492.587
Standard error	46.418	28.575	43.288
Positive	10.000	13.000	11.000
Positive (%)	66.667	86.667	73.333
≥MRL	0.000	0.000	0.000
≥MRL(%)	0.000	0.000	0.000

Table 4.15: The residue of amprolium (µg/kg) in chicken samples (n=15) collected from vendors of district Kathua, J&K (UT)

Maximum residue level, amprolium			
FAO/WHO	**1000 µg/gm**	**500 µg/gm**	**1000 µg/gm**
S. No.	**Liver**	**Muscle**	**Kidney**
1	376.210	331.445	ND
2	ND	70.308	48.407
3	369.426	217.313	328.646
4	527.294	382.985	319.645
5	386.233	324.993	424.127
6	383.301	400.005	206.951
7	325.206	359.423	399.756
8	306.497	256.309	344.797
9	320.994	165.343	358.118
10	344.433	306.467	310.369
11	67.091	45.998	0.000
12	398.221	351.201	371.111
13	306.024	227.067	43.192
14	101.725	152.546	0.000
15	360.345	225.132	310.114
Mean	304.867	254.436	231.015
Minimum residue concentration	ND	45.998	ND
Maximum residue concentration	527.294	400.005	424.127
Standard error	36.272	28.483	42.187
Positive	14.000	15.000	12.000
Positive (%)	93.333	100.000	80.000
≥MRL	0.000	0.000	0.000
≥MRL(%)	0.000	0.000	0.000

Table 4.16: The residue of amprolium (µg/kg) in chicken samples (n=15) collected from vendors of district Reasi, J&K (UT)

Maximum residue level, amprolium			
FAO/WHO	**1000 µg/gm**	**500 µg/gm**	**1000 µg/gm**
S. No.	**Liver**	**Muscle**	**Kidney**
1	ND	346.087	ND
2	289.388	393.296	394.126
3	179.648	61.476	143.085
4	277.758	308.725	297.683
5	245.202	206.298	291.243
6	ND	83.601	ND
7	203.252	113.860	266.081
8	0.341	32.662	0.000
9	203.346	149.371	205.380
10	204.545	151.057	193.892
11	238.559	114.184	160.022
12	141.059	ND	128.099
13	275.146	ND	161.656
14	221.086	132.846	ND
15	302.282	110.131	221.249
Mean	185.441	146.906	164.168
Minimum residue concentration	ND	ND	ND
Maximum residue concentration	302.282	393.296	394.126
Standard error	27.145	30.965	31.822
Positive	13.000	13.000	11.000
Positive (%)	86.667	86.667	73.333
≥MRL	0.000	0.000	0.000
≥MRL(%)	0.000	0.000	0.000

Table 4.17: The residue of amprolium (µg/kg) in chicken samples (n=15) collected from vendors of district Samba, J&K (UT)

Maximum residue level, amprolium			
FAO/WHO	**1000 µg/gm**	**500 µg/gm**	**1000 µg/gm**
S. No.	**Liver**	**Muscle**	**Kidney**
1	ND	96.095	ND
2	168.091	ND	ND
3	163.181	ND	54.973
4	290.764	196.001	141.074
5	347.362	223.968	262.159
6	223.477	145.662	148.358
7	ND	94.354	ND
8	178.646	80.381	193.919
9	122.299	147.022	164.337
10	192.361	288.382	109.870
11	120.566	79.672	195.598
12	188.860	145.608	160.890
13	74.218	0.000	0.000
14	277.381	139.928	197.499
15	76.470	150.515	0.000
Mean	161.578	119.173	108.578
Minimum residue concentration	ND	ND	ND
Maximum residue concentration	347.362	288.382	197.499
Standard error	25.893	21.373	23.569
Positive	13.000	12.000	10.000
Positive (%)	86.667	80.000	66.667
≥MRL	0.000	0.000	0.000
≥MRL(%)	0.000	0.000	0.000

CHAPTER-V

Discussion

CHAPTER-V

DISCUSSION

5.1 Sulphaquinoxaline

5.1.1 Detection and quantitation limit

The LOD and LOQ for sulphaquinoxaline were quantified as 15.427 ng/ml and 46.749 ng/ml, respectively, which is well below the prescribed MRL for the liver, kidney, and muscle samples.

5.1.2 Recovery of sulphaquinoxaline

It can be vouched from the result that the liver and kidney exhibited relatively lesser recoveries than muscles. Relatively poor recovery of sulphaquinoxaline has been reported in various studies earlier. For example, the recovery of sulphaquinoxaline from liver and kidney was 72-96%, with relative standard deviations ranging between 3 to 11% reported by Bogialli *et al.* (2003) using liquid chromatography and mass spectrometry. This poor recovery of sulphaquinoxaline from bovine liver and kidney tissue was attributed to rapid enzymatic oxidation. In addition, Bogialli *et al* further concluded that it formed a compound characterized by a molecular mass 16Da bigger than the parent compound, synthesized during sample preparation due to enzymatic oxidation. Later, Hoff *et al.* (2012) studied the characterization and estimation of sulphaquinoxaline metabolites in animal tissues using LCMS. They noticed a total loss of sulphaquinoxaline in spiked liver tissue samples of horses, and attributed it to the active enzymatic activity due to cytochrome P450 and reductase activity (Aerts *et al.*, 1995). Further, Hoff *et al* (2012) noticed that when microsomal enzyme inhibitor like potassium cyanide was added to the liver sample, spiked with sulphaquinoxaline, exhibited normal recovery and samples without microsomal enzyme inhibitor led to complete absence of the sulphaquinoxaline. This explains the relative loss of recovery in the analyte when spiked before extraction.

5.1.3 The presence of sulphaquinoxaline residues

Sulphaquinoxaline and amprolium are widely used antimicrobials in the poultry industry, to manage poultry diseases and or aimed for growth promotion. The

indiscriminate use of these drugs, including failure to observe withdrawal periods, is known to result in harmful detectable residues in chicken tissues (Tajick and Shohreh, 2006). Therefore, in the wake of the current study's design, chicken tissue (liver, kidney and muscle) samples were gathered from five districts of Jammu region, which included Jammu, Reasi, Kathua, Udhampur, and Samba.

In the present study, sulphaquinoxaline was assessed and quantified by reverse phase HPLC UV as per Cheong *et al.* (2010) with suitable modification. Cheong *et al.*, (2010) used binary gradient mobile phase with 0.01M ammonium acetate (pH 4.6, mobile phase A) and acetonitrile as mobile phase B for chromatographic separation of sulphaquinoxaline employing a UV/VIS detector (266 nm). The retention behavior of sulphaquinoxaline was dependent on its ionization and polarity of the mobile phase. Therefore, the pH of the mobile phase plays a vital role in chromatographic separation (Wang *et al*., 2006).

Out of 20 samples collected from district Jammu, 14 of the liver, 2 of kidney, and 5 of muscle were over MRL. All twenty representatives of the liver gathered from Udhampur exhibited values of sulphaquinoxaline residue beyond the prescribed MRL. Although, neither kidney nor muscles samples showed deposition beyond MRL. Among the 20 liver samples, ten samples collected from district Samba, five from Reasi, and nine from Kathua, Jammu showed sulphaquinoxaline residue beyond established MRL. Neither the kidneys nor muscles collected from Samba, Reasi, or Kathua showed any sample beyond MRL.

The reason for the prominence of sulphaquinoxaline residue in liver tissue may be due to its use in excess by the local poultry farmers, which might have impacted and overwhelmed the normal liver function. Our study results are contrary to Hoff *et al.* (2012), who stated that sulphaquinoxaline undergoes enzymatic degradation. Such findings may be due to the use of the said drug at the regular dosing, as the dose has not been mentioned in said paper. Though, Cheong *et al.* (2010) detected sulfonamides in chicken liver samples in the range of 8 to 193 ng/kg. The residues of sulphonamide were beyond MRLs (100 ng/gm) in chicken liver collected from Johor states of Malaysia, which was attributed to inadequate withdrawal period before slaughter. Further, Cheong

explained that the liver plays a significant role in the body's metabolism and has multiple functions, including plasma protein synthesis, glycogen storage, and drug inactivation. That may be a reason for high sulfonamide residues in liver samples as compared to other parts of the chicken (Cheong *et al.,* 2010).

India lacks regulations to control antimicrobials and or sales thereof for their use in the poultry sector. India is working upon and yet to come up with comprehensive rules in this regard.

Compared to those reported in other countries, the sulfonamides detected in chicken samples in several districts of Jammu region, in general, and can be considered very high. The author could not find any reports regarding the presence of sulfonamides as residues in food of animal origin in Jammu, India. Sahu and Saxena (2014) analyzed 70 samples to detect oxytetracycline, chlortetracycline, doxycycline, enrofloxacin, and ciprofloxacin in chicken in Delhi and NCR region but not sulfonamides. Waghmare *et al*. (2020) analysed over ninety chicken samples, collected from local poultry processing units at Mumbai, India and found 9.5% of muscle and 5.26% of the liver tissues positive for the residue of doxycycline. Sarker *et al.* (2018) studied chicken meat, excluding sulfonamides, in Bangladesh and found liver samples primarily positive for antibiotics residue. Antimicrobials residues have been found relatively low for various antimicrobials, including Sulfonamides in Malaysia (Cheong *et al.,* 2010). The contamination rates of sulfonamides in chicken tissues in the USA have been over 4% (Dey *et al.,* 2003). Weiss *et al.* (2007) studied and found that the residue of sulfadiazine and sulphaquinoxaline ranged from 0.64 – 21 µg/kg and 0.98-116 µg/kg, respectively, in poultry meat in Italy. The violative samples were less than 1% but always detected in the liver. Kabir *et al*., (2004) have reported contamination of sulfonamides, *i.e.*, in 1% eggs, 33.1% broilers, 23.6% slaughtered chicken, and 4.8% local birds in Nigeria. In the present study, it is inferred that the high occurrence of sulphaquinoxaline residues in Jammu might have been due to either non-adherence to the withdrawal period (FDA, 2016) or excess dosing of the sulphaquinoxaline by the local poultry farmers. It may be studied further to pinpoint the actual cause of high residues occurrence.

5.2 Amprolium

5.2.1 Detection and quantitation limit

The detection (LOD) and quantitation (LOQ) limit for amprolium were calculated using area under curve and respective known concentrations of the standard solutions, as low as 7.81ng/ml. The LOD and LOQ calculated were 145ng/ml and 441ng/ml, respectively, which were well below the prescribed MRL for amprolium residue.

5.2.2 Recovery of amprolium

The method was confirmed as per the criteria specified by EUCD 2002/675/EC (2002) to quantitate the analyte, validated by fortifying blank chicken tissue at 400 ng/gm. The recovery of amprolium were 68.316, 71.029 and 72.156% in kidney, muscles and the liver respectively. Similar figure of recovery for amprolium were reported by Yamamoto and Kondo, (2001).

5.2.3 The presence of amprolium residues

It can be vouched from the result that out of the 15 samples each, ten of the liver, 13 of muscles, and 11 of kidney collected from Jammu district were positive for amprolium residue. Similarly, out of 15 samples each, 14 of the liver, 15 of muscles, and 12 of kidney from Kathua district, Jammu was positive for amprolium residues. Likewise, out of 15 each, 13 of the liver and muscles and 11 of kidneys collected from Reasi district, Jammu were positive for amprolium residues. Similar results were also noticed from the Samba district, Jammu J&K (UT).

The residue of amprolium was detected but was no way close to the prescribed MRL for liver (1000 ng/gm), kidney (1000 ng/gm), and muscles (500 ng/gm) samples collected from various districts of the Jammu region. Although, Furusawa (2002) could not detect amprolium as residue in chicken tissues procured from Oshaka, Japan. Furusawa, further this non-detection of amprolium is due to its limited to the gastrointestinal tract. Takahashi *et al.* (1994) had observed that the amprolium exhibits low concentration despite high dosing, although not quote any reason thereof.

CHAPTER-VI

Summary and Conclusions

CHAPTER-VI

SUMMARY AND CONCLUSIONS

Sulphaquinoxaline and amprolium are commonly and widely used anticoccidials in the poultry sector in the Jammu region of J&K (UT). The typical broiler producer usually increases the dose of anticoccidials progressively to suppress the symptoms in consequent years of rearing chicken and production in the Jammu region. In this purview, this study was designed to evaluate twenty chicken samples constituting liver, kidney, and muscle in each for the residue status of sulphaquinoxaline in five districts of Jammu *viz.* Jammu, Kathua, Reasi, Samba, and Udhampur. Similarly, 15 samples each from four districts of the Jammu region, *viz.* Jammu, Kathua, Reasi, and Samba were collected to evaluate the residue profile for amprolium.

6.1 Chromatographic conditions

The chromatographic separation of sulphaquinoxaline was achieved using binary gradient mobile phase *i.e.*, 0.01M ammonium acetate (pH 4.6) and acetonitrile (43:57), with a peak retention time of 3.813 minutes.

The chromatographic separation of amprolium was attained, employing isocratic mobile phase composed of phosphate buffer (pH 5) and acetonitrile (30:70) at a peak retention time of 2.663.

6.2 Detection and quantitation limit and recovery of analyte

The LOD and LOQ for sulphaquinoline were 15.427 ng/ml and 46.749 ng/ml, respectively. After fortification of the respective tissues at 0.3 µgm/gm, the percent recovery achieved for sulphaquinoxaline in kidney, liver, and muscle tissues were 78.9 ± 0.134, 77.484 ± 0.313, 86.838 ± 0.050, respectively.

The LOD and LOQ of amprolium attained were 145 ng/ml and 441 ng/ml, respectively. The percent recovery in the liver, kidney, and muscle tissues were 72.156 ± 0.704, 68.316 ± 1.495, 71.029 ± 0.575, respectively, after the spiking of the respective tissue samples by adding 0.4 µg/gm of amprolium.

The samples were subjected to analysis following sample preparation and cleanup to injection in HPLC system. Based on the findings of the current study, the following observations were made:

a. All the samples of the liver and kidney, and a majority (90%) of muscles collected from the sampling region of Jammu district were positive, and 70 percent of the samples of liver, 10 % of kidney and 25% of muscles, had higher residue than established international MRL.

b. Out of the 20 samples, 75% of kidney and 80% of muscle tissues gathered from Udhampur were positive for sulphaquinoxaline, and none of the kidney and muscles tissues exhibited residues over MRL.

c. All liver samples gathered from Udhampur exhibited residue of sulphaquin oxaline beyond the established MRL of 100 µg/kg.

d. The liver, kidney, and muscles samples gathered from Samba were positive to the extent of 90%, 85%, and 55%, respectively. However, 50% of the liver tissues crossed the prescribed MRLs, other tissues (kidney and muscles) had residues well below established MRL values.

e. The liver, kidney, and muscles samples gathered from Reasi were positive to the extent of 85%, 65%, and 70%, respectively. Albeit, 25% of the liver tissue had sulphaquinoxaline residue over the established MRL.

f. The majority of the collected samples from Kathua were positive for sulphaquinoxaline residue. However, 45% of the liver samples had residue beyond the established MRL.

g. Out of the 15 samples collected from Jammu district, 66.66% of the liver, 73.33% of kidney, and 86.66% of muscles were positive for residue of amprolium.

h. The samples gathered from Kathua were positive for amprolium residues to the extent of 93.33%, 80%, and 100% of liver, kidney, and muscles, respectively.

i. The tissues of the liver, kidney, and muscles collected from Reasi, Jammu were found positive to the extent of 86.66%, 73.33%, and 86.66%, respectively.

j. It was found that 86.66%, 66.66 %, and 80% of the gathered samples of liver, kidney, and muscles, respectively, from district Samba, were positive for the residue of amprolium.

k. Even though, samples from all the districts under study were highly positive for amprolium residues, none of the tissues exhibited values beyond established international MRL.

Conclusion

Out of chicken samples gathered from Jammu district, 70% of liver, 10% of kidney and 25% of muscle had sulphaquinoxaline residues higher than established MRL. The values obtained regarding chicken liver samples beyond established MRL for sulphaquinoxaline from Udhampur, Samba, Reasi and Kathua were found to be 100, 50, 25 and 45% respectively. The MRL value for sulphaquinoxaline obtained for the samples of kidney and muscle from Udhampur, Samba, Reasi and Kathua were less than established MRL.

The samples gathered from Jammu, Kathua, Samba and Reasi were positive for residue of amprolium but exhibited lesser values as compared to established value of MRL.

References

REFERENCES

Aerts, M. M. L., Hogenboom, A. C. and U. A. Th. Brinkman, 1995. Analytical strategies for the screening of veterinary drugs and their residues in edible products. *Journal of Chromatography B: Biomedical sciences and applications,* **667**(1):1-40.

Alhaji, N.B., Haruna, A.E., Muhammad, B., Lawan, M.K., and Isola, T.O. 2018. Antimicrobials usage assessments in commercial poultry and local birds in North-Central Nigeria: Associated pathways and factors for resistance emergence and spread. *Preventive veterinary medicine*, **154**:139-147.

Appelgate, J. 1983. *Clinical pharmacology of sulfonamides*, Modern veterinary practice (USA).

Atta, A.H., el-Zeni and Samia, A. 1999. Tissue residues of some sulphonamides in normal and Eimeria steidai infected rabbits. *Dtsch Tierarztl Wochenschr*. **106** (7): 295-298.

Ahmad, A. H., Geetanjali and Pant Disha. 2019. Pharmacokinetics of sulfaquinoxaline following single dose oral administration in poultry. *Journal of Veterinary Pharmacology and Toxicology*, **18** (1):44-47.

Banerjee, I., and NC, K. 1974. Distribution of sulfaquinoxaline in tissues of poultry. *Ind J. Physiol. Pharmac*, **18**: 361-363.

Barragry, T.B. 1994. *Veterinary drug therapy.* Lea and Febiger, Philedelphia, USA.

Biswas AK, Rao GS, Kondaiah N, Anjaneyulu AR, Mendiratta SK, Prasad R 2007. A simple multi-residue method for determination of oxytetracycline, tetracycline, and chlortetracycline in export buffalo meat by HPLC-photodiode array detector. *J. Food Drug Anal*, **15**(3): 278-284.

Basic Animal Husbandry Statistics – 2019. Government of India. *Ministry of Fisheries, Animal husbandry and Dairying, KrishiBhawan*, New Delhi.

Beyene, T. 2016. Veterinary drug residues in food-animal products: Its risk factors and potential effects on public health, *Journal of Veterinary Science and Technology*, **1**(7):285.

Botsoglou, N.A., and Fletouris, D.J. 2001. *Drug residues in foods.* Marcel Dekker, New York, USA.

Byung-Ju Kim, Hyun-Sun Ham, Jin-Joo Lee, Nam-Yong Cheong, Seung-Woon Myung. 2012. Determination of coccidiostats (amprolium and decoquinate) in cattle and chicken's muscle using high performance liquid chromatography. *Bulletin of the Korean Chemical Society*, 33**(2)**: 559-563.

Brown, G.M. 1989. The biosynthesis of folic acid II. Inhibition by sulfonamides. *J Biol Chem.* **237**: 536-540.

Bogialli, S., Curini, R., Di Corcia, A., Nazzari, M., and Sergi, M. 2003. Confirmatory analysis of sulfonamide antibacterials in bovine liver and kidney: extraction with hot water and liquid chromatography coupled to a single-or triple-quadrupole mass spectrometer. *Rapid communications in mass spectrometry*, **17**(11): 1146-1156.

CAC, Codex Alimentarius Commission 2011. Maximum residue limits for veterinary drugs in food. *CAC, Rome*, pp, 36

Cheong, C.K., Hajeb, P., Jinap, S., and Ismail-Fitry, M.R. 2010. Sulfonamides determination in chicken meat products from Malaysia. *International Food Research Journal*, **17**:885-892.

Chitescu, C.L., Nicolau, A.L., Csuma, A., and Moisoiu, C. 2011. Simultaneous analysis of four sulfonamides in chicken muscle tissue by HPLC. *Food Additives & Contaminants.*Part A, **28**(8): 1013-1020.

Choquet-Kastylevsky, G., Vial, T., and Descotes, J. 2002. Allergic adverse reactions to sulfonamides. *Current Allergy and Asthma Reports*, **2**(1): 16-25.

Code of Federal Regulation Title 21, USFDA 2020. Department of health and human service subchapter E- Animal drugs feeds and related products. Part 556 tolerances for residues of new animal drugs in food.

Collins, B.K., Moore, C.P., and Hagee, J.H. 1986. Sulfonamide-associated keratoconjunctivitis sicca and corneal ulceration in a dysuric dog. *Journal of the American Veterinary Medical Association*, **189**(8): 924-926.

Conway, A. 2017. Meat Production: Poultry meat production up 13 million metric tons by 2026. Fig. 2: Global meat production by species. *Poultry Trends*: 22-23.

Daft, B.M., Bickford, A.A., and Hammarlund, M.A. 1989. Experimental and field sulfaquinoxaline toxicosis in leghorn chickens. *Avian Diseases*, **33**: 30-34.

Desimoni E., and Brunetti, B. 2015. About estimating the limit of detection by the signal to noise approach. *Pharm Anal Acta, **6***: 355.

Dey, B. P., Thaler, A. and Gwozdz, F. 2003. Analysis of microbiological screen test data for antimicrobial residues in food animals. *Journal of Environmental Science and Health Part B,* **38**(3):391-404.

Donoghue, D. J. 2003. Antibiotic residues in poultry tissues and eggs: human health concerns. *Poultry science*, **82**(4): 618-621.

EC, European Commission 2002. Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results *J. Eur. Communi.,***221**: 8-36.

El-Kholy, H., & Kemppainen, B. W. 2005**.** Levamisol residues in chicken tissues and eggs. *Poultry Science,* **84**(1): 9-13.

El-Sayed, M.G., Abd El- Aziz, M.I. and El- Kholy, M.H.1995. Kinetic behavior of sulphaquinoxaline and amprolium in chickens. Dtsch. Tierarztl. Wochenschr. **102**(12): 481-485.

Engberg, R.M., Hedemann, M.S., Leser, T.D., Jensen, B.B**.** 2000 .Effect of zinc bacitracin and salinomycin on intestinal microflora and performance of broilers. *Poult Sci*, **79**(9): 1311-1319.

European Commission 2010. Commission Regulation (EU) No. 37/2010 of 22 December 2009: on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. *Official Journal of the European Union*, L**15**, 1-72.

Evanthia, P.., Tolika, Victoria, F., Samanidou and Ioannis, N.P.2012. Determination and validation of an HPLC method for the simultaneous determination of ten sulfonamide residues in bovine, porcine and chicken tissues according to 2002/657/ EC. *Current Pharmaceutical Analysis,* **8**(1): 56-67.

FAO/WHO 1995. Application of risk analysis to food standards issues. *Report of a Joint FAO/WHO Expert Consultation*, 13-17 March 1995, Geneva, WHO/FNU/FOS/ 95.3, World Health Organization, Geneva.

FAO/WHO 2008. Definitions for the purposes of the Codex Alimentarius In: Procedural manual, 18th ed. Rome, Food and Agriculture Organization of the United Nations, *Codex Alimentarius Commission*, p 17-19.

Furusawa, N. 2002. Simplified high performance liquid chromatographic determination of residual amprolium in edible chicken tissues. *Journal of Chromatographic .Science*, **40**(6): 355-358.

Furusawa, N., and Kishida, K. 2002**.** Transfer and distribution profiles of dietary sulfonamides in the tissues of the laying hen. *Food Addit. Contam.,* **19**(4): 368-372.

Food and Drug Administration, HHS 2016. New Animal Drugs for use in Animal Feed; Approval of New Animal Drug Applications; Withdrawal of Approval of New Animal Drug Applications. Federal Register: *The Daily Journal of the United States Government.*

Hamamoto, K., Koile, R., Shirakuru, A., Sasaki, N., and Machida, Y. 1997. Rapid and sensitive determination of amprolium in chicken plasma by HPLC with post-column reaction. *Journal of Chromatography B,* **693**: 489-492.

Hamid, A. N. 2012. *Country Report*: Malaysia Organization of the department of veterinary services, pp. 1-8.

Hela, W., Brandtner, M., Widek, R., & Schuh, R. 2003. Determination of sulfonamides in animal tissues using cation exchange reversed phase sorbent for sample cleanup and HPLC–DAD for detection. *Food Chemistry,* **83**(4): 601-608.

Hoff, R.B., Barreto, F., Melo, J., Jank, L., Peralba, M. D. C. R., & Pizzolato, T. M. 2012. Characterization and estimation of sulfaquinoxaline metabolites in animal tissues using liquid chromatography coupled to tandem mass spectrometry. *Analytical Methods,* **4**(9):2822-2830.

Hormabazal V. and Ynstead M. 2000. Rapid assay for the determination of residues of amprolium and ethopabate in chicken meat by HPLC. J. Liq. Chromatogr. **23**: 1585-98.

ICRA 2014. Indian Poultry Industry- Broiler meat and Table eggs, April 2014, http://www.icra.in/ticker/SH-2014-1-ICRA- poultry pdf.

Jackson, B.A.1990. US Food and Drug Administration, Center for Veterinary Medicine. *Hosted at University of Nebraska Lincoln. J. Vet. Med. Assoc.,* **3**(2): 176-196.

Jain, S.K. 1994. Studies on metabolism and disposition kinetics of sulfonamides in sheep.

Jensen, J., Krogh, P.H., & Sverdrup, L.E. 2003. Effects of the antibacterial agents tiamulin, olanquindox and metronidazole and the anthelmintic ivermectin on the soil invertebrate species Folsomiafimetaria (Collembola) and Enchytraeus crypticus (Enchytraeidae). *Chemosphere,* **50**(3): 437-443.

Kabir, J., Umoh, V., Andu-Okoh, E., Umoh, J. ., & Kwaga, J.K. 2004. Veterinary drug use in poultry farms and determination of antimicrobial drug residues in

commercial eggs and slaughtered chicken in Kaduna State, Nigeria. *Food Control,* **15**(2): 99-105.

Kahn, C.M., Line, S., and Aiello, S.E. 2005**.** *The merck veterinary manual*, Merck & Co. Inc., Whitehouse station, NJ.

Kao, Y., Chang, M., Cheng, C., & Chou, S. 2001. Multiresidue determination of veterinary drugs in chicken and swine muscles by high performance liquid chromatography. *Journal of Food and Drug Analysis,* **9**(2): 84-95

Kart, A., and Bilgilli, A. 2008. Ionophore antibiotics: toxicity, mode of action and neurotoxic aspect of carboxylic ionophores. *J Anim Vet Adv,* **7**(6): 748-751.

Khishida, K., Furusawa, N. 2003. Toxic/Harmful solvents-free technique for HPLC determination of six sulfonamides in meat. *Journal of liquid chromatography & related technologies,* **26** (17): 2931-2939.

Kietzmann, M. 1980. Beitrag zur Pharmakokinetik von Sulfonamiden beim Geflugel. (Doctoral dissertation, Uitgever niet vastgesteld).

Kirbis, A. 2007. Microbiological screening method for detection of aminoglycosides, β-lactames, macrolides, tetracyclines and quinolones in meat samples. *Slovenian Veterinary Research,* **44**(1/2): 11-18.

Kolar, M., Urbanek, K., and Latal, T. 2001. Antibiotic selective pressure and development of bacterial resistance. *International journal of antimicrobial agents*, **17**(5): 357-363.

Leeuwen, Van, W., and Wilhelmus van Gend, H. 1988. Determination of amprolium in egg yolk and muscle tissue (chicken) by HPLC with post-column reaction and fluorometric detection, using on-line sample clean-up and pre-concentration steps. *Zeitschrift fur Lebensmittel-untersuchung und-forschung*, **186**(6): 500-504.

Li, T., Qiao, G.L., Hu, G.Z., Meng, F.D., Qiu ,Y.S., Zhang, X.Y., Guo, W.X., Yie, H.L., Li, S.F., Li, S.Y. 1995. Comparative plasma and tissue pharmacokinetics and drug

residue profiles of different chemotherapeutants in fowls and rabbits. *Journal of Veterinary Pharmacology and Therapeutics,* **18**(4), 260-273.

Liu, C.K. 2011. Caution with animal drugs to prevent food safety. *Veterinarian Newsletter,* **2**: 15-17

Long, P.L. 1963. The effect of a combination of sulfaquinoxaline and amprolium against different species of Eimeria in chickens. *Vet. Rec*, **75**: 645-650.

Leoni, V., Caricchia, A.M. and Chlavarine, S. 1992. Multi-residue method for quantitation of organophosphate pesticides in vegetable and animal food. *J. AOAC International*, **75**: 511-518.

Mathis, G.F., McDougald, L.R.,and Mcmurray, B.I.R.C.H. 1984. Effectiveness of therapeutic anticoccidial drugs against recently isolated coccidian. *Poultry Science*, **63**(6): 1149-1153.

Mehtabuddin, A. A.Mian, T. Ahmad, S. Nadeem, Z. I. T., and J.A. 2012. Sulphanamide residues determination in commercial poultry meat. *The Journal of Animal & Plant Sciences*, **22**(2): 473-490.

Mundi, I. 2015. India Broiler Meat (Poultry) Production by Year.

Mantri, A.P., Mubeen, R.S., Kandepu, N., and Kumar, S. 2015. Simultaneous estimation of sulfaquinoxaline sodium and amprolium hydrochloride by RP-HPLC. *International Journal of Pharmaceutical Sciences and Research*, **6**(3): 1097-1100.

Nagata, T., and Saeki, M. 1986. Determination of ampicillin residues in fish tissues by liquid chromatography. *Journal of the Association of Official. Analytical. Chemists,* **69** (3): 448-450.

Neer, T.M., and Savant, R.L. 1992. Hypoprothrombinemia secondary to administration of sulfaquinoxaline to dogs in a kennel setting. *JAVMA.***200** (9): 1344-1345

Nouws, J.F.M., Geertsma, M.F., Grondel, J.L., Aerts, M.M.L., Vree, T.B., and Kan, C.A. 1988. Plasma disposition and renal clearance of sulphadimidine and its metabolites in laying hens. *Res. Vet. Sci*.**44**: 202-207

Oikawa, Y., Nakamoto, K., Hirota, K., and Katagiri, K. 1977**.** Clearance of sulfomethoxazole in eggs and tissues of chickens. *Poult. Sci*.**56**: 813-821

Paige, J.C. 1994. Analysis of tissue residues. *FDA Vet,* **9**:4-6.

Patterson, J.M., and Grenn, H.H. 1975. Hemorrhage and death in dogs following the administration of sulfaquinoxaline. *Can Vet J*.**16** (9): 265-268.

Plumb, D.C. 1991**.** *Veterinary drug hand book*. White Bear lake, Minn: Pharma Vet Publ. USA.

Plumb, D.C. 2005**.** *Veterinary drug hand book*, 5th edition. Blackwell Publishing Professional, Ames, Lowa USA.

Prabakaran, R. 2014. Indian Poultry Industry- Current Status, Practical Challenges and Opportunities. In: *Proceedings of the 31st Annual Conference and National Symposium of Indian Poultry Science and Association (IPSACON 2014),* pp. 18-20, Namakkal, India.

Prajwal, S., Vasudevan, V.N., Sathu, T., Irshad, A., Nayankumar, S.R. 2017. Kuleswan Pame antibiotic residues in food animals: Causes and health effects. *The Pharma Innovation Journal*, **6**(12):1-4.

Prescott, J.F., and Baggot, J.D. 1993. Sulfonamides, trimethoprim, ormetoprim, and their combinations. *Antimicrobial Therapy in Veterinary Medicine,* **2:** 229-251.

Prescott, J.F., Baggot, J.D., and Walker, R.D. 2000. *Antimicrobials therapy in veterinary medicine*. 3rd edn. Ames, lowa, Blackwell, USA.

Rajendran, K., Mani, K., Shamsudeen, P., and Vasanthakumar, T. 2014. Broiler industry–understanding the integration and role of private industries. In: *Proceedings of the*

*31st Annual Conference and National Symposium of Indian Poultry Science Association.*pp. 18-20.

Riviere, J. E., Craigmill, A.L. and Sundlof, S.F. 1991. *Handbook of comparative pharmacokinetics and residues of veterinary antimicrobials*, Boca Raton, FL: CRC press, Inc., Florida, USA.

Rychen G *et al.,* 2018. Safety and efficacy of COXAM® (amprolium hydrochloride) for chickens for fattening and chickens reared for laying. *EFSA J*, **16**(7): 377-384.

Sabatino, M.D., Anna, P.D. M., Luigi, B., and Bruno, S.D. 2007: Determination of ten sulfonamide residues in meat samples by liquid chromatography with ultraviolet detection. *Journal of AOAC international*. **90**: 598-603.

Sahu, R., and Saxena, P. 2014.Antibiotics in Chicken Meat. *Centre for Science and Environment.*

Salisbury, C.D.C., Sweet, J.C., and Munro, R. 2004. Determination of sulfonamide residues in the tissues of food animals using automated precolumn derivatization and liquid chromatography with fluorescence detection. *Journal of AOAC International.* **87**(5):1264-8

Sandhu 2014. *Essentials of Veterinary Pharmacology and Therapeutics* 2nd edition. pp, 1081.

Sarker, Y. A., Hasan, M. M., Paul, T.K., Rashid, S. Z., Alam, M.N., &Sikder, M. H**.** 2018. Screening of antibiotic residues in chicken meat in Bangladesh by thin layer chromatography. *Journal of Advanced Veterinary and Animal Research,* **5**(2):140-145.

Simeonidou, E.J., Botsoglou, N.A., Psomas, I.E. and Fletouris, D.J. 1996. Liquid Chromatographic analysis of multiple sulfonamides residues in chicken muscle using pre-column derivatization and fluorescence detection. *Journal of Liquid Chromatography & Related Technologies.***19** (14): 2349- 2364.

Spoo, J.W., and Riviere, J.E. 2001. Sulfonamides. In: *Adams, H.R. ed. Veterinary Pharmacology and Therapeutics. IOWA State University Press. Ames*, 796-817

Stoev, G., and Michailova, A. 2000. Quantitative determination of sulfonamide residues in foods of animal origin by high- performance liquid chromatography with fluorescence detection. *Journal of Chromatography A.* **871**(1/2):37-42.

Takahashi, Y., Nishikawa, M., and Endoh, Y.S. 1994. Simultaneous high performance liquid chromatographic determination of amprolium, ethopabate, sulfaquinoxaline and N4-acetylsulfaquinoxaline in chicken tissues. *Journal of Liquid Chromatography*. **17**(20):4489-4512.

Tajick, M.A., and Shohreh, B. 2006. Detection of antibiotics residue in chicken meat using TLC. *International Journal of Poultry Science*, **5**(7): 611 - 612.

Vree T.B., and Hekster, Y.A. 1985. Pharmacokinetics of sulfonamides revisited. *Antibiot. Chemother*.**34**: 1-208.

Waghamare, R.N., Paturkar, A. M., Vaidya, V. M., Zende, R J., Kumar, A., and Bedi, J. S. 2020. Screening of antimicrobial residues and confirmation of doxycycline in samples collected from chicken farms and processing units located around Mumbai, India. *Indian Journal of Animal Research*, **54**(11): 1415-1421

Weiss, C., Conte, A., Milandri, C., Scortichini, G., Semprini, P., Usberti, R. and Migliorati, G. 2007. Veterinary drugs residue monitoring in Italian poultry: Current strategies and possible developments. *Food Control* **18**: 1068-1079.

Wang, S., Zhang, H. Y., Wang, L., Duan, Z. J., and Kennedy, I. 2006. Analysis of sulphonamide residues in edible products. A review. Food additives and contaminants, **23** (4): 362-384.

Yamamoto, Y., and Kondo, F. 2001. Determination of halofuginone and amprolium in chicken muscle and egg by liquid chromatography. *Journal of AOAC International*, **84**(1):43-46.

Zhang, Yan, and Yinliang Wu. 2005. Determination of residual sulfonamides in meat by high performance liquid chromatography with solid-phase extraction. *Se pu = Chinese journal of chromatography* **23** (6): 636-6

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CERTIFICATE – IV

Certified that all the necessary corrections as suggested by the external examiner/evaluator and the advisory committee have been duly incorporated in the thesis entitled **"Estimation and Quantification of antimicrobial residue in poultry meat available in and around Jammu region"** submitted by **Ms. Lenesha Manhas,** Regd. No. **J-19-MV-597**.

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