NEAR EAST UNIVERSITY
INSTITUTE OF GRADUATE STUDIES
DEPARTMENT OF FOOD HYGIENE AND TECHNOLOGY

DETERMINATION OF VETERINARY ANTIMICROBIAL RESIDUES IN BEEF AND POULTRY MEAT USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY MASS SPECTROMETRY AND METHOD VALIDATION

PhD THESIS

Belachew Bacha HIRPESSA

Nicosia
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Approval

We certify that we have read the thesis submitted by Belachew Bacha Hirpessa titled **"Determination of Veterinary Antimicrobial Residues in Beef And Poultry Meat Using High Performance Liquid Chromatography Mass Spectrometry and Method Validation"** and that in our combined opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Doctor of Food hygiene and technology. Thesis defence was held online. The Jury members declared their acceptance verbally which is recorded.

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Declaration

I hereby declare that all information, documents, analysis and results in this thesis have been collected and presented according to the academic rules and ethical guidelines of Institute of Graduate Studies, Near East University. I also declare that as required by these rules and conduct, I have fully cited and referenced information and data that are not original to this study.

Belachew Bacha Hirpessa

30/06/2022

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Belachew Bacha Hirpessa

Abstract

Determination of Veterinary Antimicrobial Residues in Beef and Poultry Meat Using High Performance Liquid Chromatography Mass Spectrometry and Method Validation

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Veterinary antimicrobials are mainly used in food-producing animals for therapeutic, prophylactic effects, metaphylaxis and as growth promotion purposes and may end up with the occurrence of residues in animal source food. The present study was conducted to detect and determine the levels of residues of mainly used antimicrobials in raw beef and eviscerated poultry muscle. The samples were collected from butcher shops and supermarkets found in Addis Ababa and Bishoftu cities of Ethiopia. In this preliminary work, high-performance liquid chromatography with mass spectrophotometer (HPLC-MS/MS) method was used for the analysis, samples being prepared in solid-phase extraction and purification technique. Chromatographic separation was performed using reverse phase C18 column, the mobile phase being delivered in gradient elution mode. Acquisitions of mass spectral parameters were performed in multiple reaction-monitoring (MRM) mode by a triple quadrupole mass spectrometer with an electrospray ionization technique in a positive mode. The method was optimized and validated according to the European Union (EU) commission 2002/657/EC and 2021/808 guidelines. The methods were able to quantify the antimicrobial residues with very good linearity (coefficient of determination, $r^2 > 0.99$). Relative matrix effect tests of each compounds in beef sample matrices were below 20% except for signal enhancement in doxycycline (26.03%). The accuracy ranged from 93.9 to 108.4%, repeatability was below 11% and within-lab-reproducibility ranges from 4.44 to 17.2 %. The limit of detection, limit of quantitation, decision limit (CCα), and detection capability (CCβ)

were determined for each analyte to show method sensitivity and fulfill the criteria for a confirmatory method of analysis. Based on the validation protocol and after first optimization, 180 beef samples were assessed for the presence of sulfadiazine (SDZ), oxytetracycline (OTC), tetracycline (TTC), enrofloxacin (ENR), doxycycline (DXY) and Penicillin G (PnG). Similarly using the second validation procedure, 120 eviscerated poultry meat tissues were assayed for SDZ, OTC, TTC, ENR, Sulfadimidine (SDM) and DXY. From those assays, result showed that 14.44 % and 53.33 % of beef and poultry meat samples were positive for at least one of the six antimicrobial residues assessed. From the six antimicrobial residues tested in raw beef sample matrices, OTC, TTC and SDZ account for 10.55 %, 2.78 % and 1.11% of the prevalence respectively from higher to lower level of occurrence. In eviscerated poultry meat samples, four antibiotic residues SDZ, OTC, ENR and DXY were reported at the rate of 3.33%, 20.0%, 18.33% and 11.67% respectively. Regarding residues level in the tissues, SDZ ranges from 9.25 to 13.29 µg/kg, OTC from 9.60 to 145.69 µg/kg, DXY from 10.76 to 28.5 µg/kg and ENR from 15.12 to 407.13 µg/kg. In the poultry meat samples, all residue concentrations of SDZ, and DXY quantified were below the MRLs established by either EU or Codex recommendations. Whereas, 0.8% and 6.67% of poultry samples were quantified to contain OTC and ENR above the EU MRLs separately. Eventually all the tested beef samples detected to be positive and samples with none-detectable levels of antimicrobial residues were safe and acceptable for human consumption. Whereas, a total of 9 (7.5%) poultry meat samples were unsafe and contain antimicrobial residues above EU MRLs and unfit for human consumption. Hence, the use of veterinary antimicrobials at the poultry production farms should respect withdrawal period of the drugs to reduce the level of antimicrobial residues in chicken meat below the tolerance level. In general, the responsible authority needs to conduct planned residue monitoring activities and strengthening of awareness-raisings endeavors. Because potential misuses might lead to a harmful level of antimicrobial residues to occur at any time.

Keywords: antimicrobials, beef, residues, mass spectrometer, method validation

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List of Abbreviations

ADI	Acceptable daily intake
ARfD	Acute Reference dose
CAC	Codex Alimentarius Commission
EU	European Union
MRL	Maximum Residue Limits,
ADI	Acceptable Daily Intake
AJS-ESI	Agilent jet stream electrospray ion source
CAC	Codex Alimentarius Commission
CID	Collision-induced dissociation
CRLs	Community Reference Laboratories
CVM	Center for Veterinary Medicine
EC	Commission of the European Union
EFSA	European Food Safety Authority
ELISA	Enzyme Linked Immuno Sorbent Assay
EMA	European Medicines Agency
ESI	Electrospray ionization
FAO	Food and Agriculture Organization of the United Nations
FDA	Food and Drug Administration of the USA
FSIS	Food Safety Inspection Service
HLB	Hydrophilic-Lipophilic Balance
HPLC-UV/DAD	High Performance Liquid Chromatography coupled to Ultraviolet-Visible Diode Array detector
JECFA	The Joint FAO/WHO Expert Committee on Food Additives
ACN	Acetonitrile
MRLs	Maximum Residue Limits
MRM	Multiple reaction monitoring
NMPF	National Milk Producers Federation
OIE	World Organization for Animal Health (OIE)

PBP	penicillin-binding proteins
PRiME	Process Robustness improving Matrix effect
RMP	Residue Monitoring Program
RP	Reverse Phase
SPE	Solid Phase Extraction
TLC	Thin Layer Chromatography
UHPLC	Ultra-High Performance Liquid Chromatography
USDA	United States Department of Agriculture
WHO	World Health Organization
WLR	Within-Laboratory Reproducibility
WTO	World Trade Organization

CHAPTER I
Introduction

Veterinary antimicrobials are usually administered in farmed animals kept for food production for disease treatment, prevention, control and for growth augmentations. Besides, they play crucial roles in keeping animals' health and welfare (Mensah et al., 2014; Wall et al., 2016). Owing to their growth-promoting effects, antibiotics are also used at sub-therapeutic doses in feed for extended periods to improve feed efficiency and to make animals reach marketable weight fast for economic advantages (Mensah et al., 2014; Padol et al., 2015).

Consequently, the treatment of these farmed animals with antimicrobial agents leaves residues of veterinary antimicrobials or their active byproducts in the tissues and the food derived from them may end up entering consumer's food chain (Wassenaar, 2005). Much residue concentrations could occur in the animal source food (ASF) and their products either due to extra-label drug use or due to failure to comply with withdrawal periods and poor livestock production practice (Jeong et al., 2010; Song et al., 2016).

Worldwide, due to occurrences of unsafe levels of antimicrobial residues in ASF, there has been an increasing public health concern. The worry is due to adverse health effects on consumers such as allergic reactions (Baynes et al., 2016; Zhang et al., 2021) and alteration of intestinal micro-flora by eliminating susceptible strains (Kim et al., 2017). The most serious concern is the development and spread of antimicrobial-resistant microbial strains (Ventola, 2015).

When food source animals are slaughtered or their edible products are collected before dispatch, there is a legal requirement that antimicrobial concentrations in the products should not be greater than the safe levels set by the national regulatory authority of the product origin. In many countries, this higher concentration is termed to as maximum residue limits (MRLs) or tolerance. MRLs are established based on various

factors but the major determining factor is food safety (Riviere and Sundlof, 2009; Lee et al., 2018). For instance, for effective containment of residues in ASF, European Union (EU) countries have restricted the nontherapeutic applications of veterinary antimicrobials in animals used for food production and have placed MRLs for veterinary antimicrobials in edible food matrices such as meat (EC, 2010)

Currently, developing countries, which have livestock resources, are facing difficulties to compete in the market because of increasingly stringent safety and quality standard requirements by the importing countries. Developing countries like Ethiopia to penetrate more into the international market of animal-originated food and obtain their market share, they should work on setting standards and establishing a quality assurance system (Moreno and Lanusse, 2017). To assess the level of antimicrobial residues in ASF and come up with data evident enough for designing strategies to minimize residue exposure, there should be reliable and gold standard assaying methods that measure values at trace level (Delatour et al., 2018).

However, in Ethiopia there is a lack of sufficient data regarding the presence of drug residues in ASF and their products, there are no research work done using a reliable and standard method. Researches that had been reported so far were performed using HPLC-DAD method and the Premi test (Addisalem and Bayleyegn, 2012; Agmas and Adugna, 2018). The test methods employed were not enough sensitive and may provide false-positive results. There was no study about antimicrobial residues detection and quantification on sample matrices of animal source food using ultra-high-performance liquid chromatographic instrument coupled with a triple quad mass spectrometer (UHPLC-MS/MS) and sample preparation technique using solid-phase extraction method.

Therefore, this recent research was conducted to know the levels of residues of mainly used veterinary antimicrobials in ready-to-eat beef muscle and eviscerated poultry meat tissue samples collected from butcheries and supermarkets found in two selected cities of Ethiopia. The research was designed to detect residues of commonly

used seven veterinary antimicrobials, know their level of occurrence, quantify the concentrations in beef and eviscerated poultry meat and assess the safety level of such food sources from antimicrobial residues perspective with MRLs compliances or non-compliances. In this preliminary work, screening and confirmatory method of multi-drug residues analysis by UHPLC-MS/MS, from the United States-Food Safety and Inspection Service (FSIS, 2013), was optimized and validated in the house for assaying of sulfadiazine (SDZ), sulfadimidine (SDD), oxytetracycline (OTC), tetracycline (TTC), enrofloxacin (ENR), doxycycline (DXY) and penicillin G (PnG) in meat matrices.

Statement of the problem

Different classes of antimicrobials are in use for disease treatment and prevention purposes in food animal husbandry practices. From these antimicrobials, oxytetracycline, doxycycline, amoxicillin, enrofloxacin, colistin sulfate, sulfonamides, gentamycin, streptomycin, tylosin mainly used in livestock production practice (Darwish et al., 2013, OIE, 2015). Reports from researches done on veterinary drug use and abuse in Ethiopia (Beyene et al., 2015, Gemeda et al., 2020) and personal communications and observations made indicated that, producers are using these antibiotics as prophylactic therapy in the prevention and control of diseases, to control environmental stresses factors, which might causes loss of productions, and to cover faulty management defects. Failure of prudent use of these antimicrobials and problem of making wise considerations and lack of precaution while making therapeutic decisions (overdosing and inability to stick the withdrawal period) will lead to residue in the body of food treated farm animals. The presence of residues in edible animal origin food affect their quality and safety from such sources. This directly influences the consumer's food safety in general and reduces country's competitiveness in the international trade of animal originated food.

In Ethiopia, specifically poultry production are currently expanding and there is an intensive but less regulated and uncontrolled use of antimicrobials. Hence, these antimicrobial agents are entering in to the human food chain and pausing food safety and quality hazards (Etefa et al., 2021). Hence, the below mentioned research questions need

to be studied and the extent of the problems related to residues, have to be scientifically assessed.

General and specific objectives

The principal objectives of this research projet was to detect and determine residue levels of commonly used veterinary antimicrobials in ready-to-eat beef and eviscerated poultry meat muscle using a validated method and assess the safety level of such food sources from antimicrobial residues perspective in connection with MRLs compliances or non-compliances.

Specific objectives

Therefore, the strategic specific objectives of this study were to:

- Validate the selected multiclass antimicrobial residue analysis method as per international standards and use it for assaying in beef and poultry meat samples
- Detect residues of mainly used veterinary antimicrobials and know their level of occurrence in samples collected from the study area (Oxytetracycline, Tetracycline, Doxycycline, Penicillin-G, Enrofloxacin, Sulfadiazine and Sulfadimidine)
- Determine the level or concentration of antimicrobial residues in commercially produced raw beef and eviscerated poultry meat
- Assess the safety level of such food sources from antimicrobial residues with respect to MRLs compliances or non-compliances

Research questions

- Is the in-house validated method of residue analysis fit for the intended purpose?
- Do the validated test methods fulfill the validation criteria of EU and Codex guidelines?
- What will be the prevalence of veterinary antimicrobial residues in raw beef and eviscerated poultry meat of the study areas?
- Is antimicrobial drug residues quantified in the tissue samples are above the maximum residue limits (MRLs) or not?
- In which type of meat or meat sources high levels of antimicrobial drug residues are determined, is it in beef or chicken?

Scope of the study

The scopes of this research were validation of the selected analytical methods of antimicrobial residues analysis as per international guidelines, screening and quantification) of antimicrobial residues in raw beef and eviscerated poultry meat muscles. In the laboratory activities, qualitative (screening test) and quantitative methods/techniques was employed to collect the different data. For antimicrobial residues analysis liquid chromatography coupled with mass spectrometry (LC-MS/MS) of multi drug residues analysis was employed to quantify the actual antimicrobial residues.

Significance of the study

Currently, there is an increasing demand for safe and high quality ASF, especially with a dietary preference for white meat, like poultry meat, which is cholesterol free. Besides, the escalating consumer's awareness and recent public health concerns about residues from animal originated food and the increasing problems of antimicrobial resistance (AMR) are putting the public organizations to perform planned antimicrobial residue and AMR monitoring and evaluation activities, and related programs. However, in order to prevent and control problems related to antimicrobial residues; conducting surveillance over the prevalence, source and extent of the problems are crucial.

Therefore, this research will provide an organized data and information about the quality and safety of beef and eviscerated poultry meat from antimicrobial residues aspects. The research will also support the national consumers safety and protection endeavors and will awake the responsible bodies to work towards the prevention and mitigation strategies of the problems or to work for the persistence of a good situation (if any) through provision of data on such timely and advanced issues. In general, this research will play a role for the improvement of food safety concerns and delivers preliminary data and knowledge to the scientific community on the issue. It provides data to organizations that work in the area of food safety, in prevention of the public

health, for policy makers, regulatory bodies and legislators. Moreover, this study will make data available for further research to be conducted at large scales.

CHAPTER II

Literature Review

Veterinary antimicrobials are one of the widely used classes of veterinary medicinal products (VMPs), in farmed animals' practices. The primary and rational use of antimicrobials in food producing animals is for treatment and prevention of diseases. Positively for the treatment of different infectious bacterial diseases like mastitis, inflammatory, respiratory infections and gastrointestinal diseases, (Darwish et al., 2013, OIE, 2015). In addition, antimicrobials are used prophylactically to prevent diseases for which vaccines are not available or effective. However, in current farming practices, antimicrobials are specifically used for prophylactic and metaphylactic means of diseases due to consequence of intensive animals rearing at high stocking densities (Dibner and Richards, 2005).

Antimicrobial Agents and Use in Food Producing Animals

The different classes of antimicrobials mainly used in food-producing farmed animals are the β-lactams, tetracyclines, aminoglycosides, lincosamides, macrolides, and sulfonamides (Table 1 and 2). These antimicrobials used, to avoid severe economic losses, unacceptable animal sufferings and the risk of widespread of epidemics (Wassenaar, 2005; OIE, 2015).

In general, treatment (curative) therapy is the indication of antimicrobial drugs to sick animals in the required high doses for a certain course of therapy in order to treat them from particular infections (Reference, 20--). Whereas prophylactic (preventive) use is the sub therapeutic use of antibiotics with the intention to prevent occurrence of diseases in advance with no manifestation of clinical signs (EMA, 2019) and to avoid secondary complications (Darwish et al., 2013; Muaz et al., 2018). Metaphylaxis use (control therapy) is group treatment of apparently infected animals by mass administration of antimicrobials, especially in the intensive production systems (poultry, pig and fish); assumed to be in contact with sick animals showing signs of a contagious

disease. It is practiced by producers to treat entire groups of animals, when the risk of being infected is considered very high, despite there being only a few affected individual animals (CVMP, 2016, EMA, 2019).

Table 1.

Antimicrobial Agents and Their Mechanism of Actions

Group of Antimicrobials	Pharmacologically active substance	Mechanism of Actions (MOAs)	Remarks (References)
Aminoglycosides	Streptomycin*, gentamicin*, DH-streptomycin*, kanamycin, neomycin, apramycin, spectinomycin	Inhibition translocation of t-RNA of bacterial protein production via attaching to the 30s ribosomal subunit of the bacterium.	
Amphenicols	Chloramphenicol^, thiamphenicol, florfenicol	Prevent formation of peptide bonds by acting on the 50s subunit.	^ Banned (CAC, 2018)
Beta-lactams	Amoxicillin*, penicillin*, ampicillin*, cephalosporin ceftiofur, cefazolin, oxacillin	Act on by interfering with the formation of the peptidoglycan layer and impair the formation of bacterial cell wall.	
Macrolides	Erythromycin*, Josamycin Spiramycin, Tylosin*, Lincomycin	Reversible binding with bacterial ribosome 50S sub-units, inhibiting the translocation of peptidyl-tRNA	
Quinolones	Ciprofloxacin*, difloxacine, enrofloxacin, flumequine, norfloxacin, Oxolinic acid,	Act by inhibiting bacterial gyrase and topoisomerase IV, inhibit nucleic acid (DNA) synthesis. Used as growth promoters*	
Sulfonamides	All substances belonging to the group*	Inhibit the synthesis of folates by the action of competitive inhibitors of dihydropteroate synthase	
Tetracyclines	Chlortetracycline* Doxycycline* Oxytetracycline* and Tetracycline*	By attaching with ribosomal 30S sub-units, inhibit binding of aminoacyl-tRNA to the mRNA ribosome complex	

*legally authorized and commonly used antimicrobials in food animal farming practice in Ethiopia, ^ Prohibited drug in food animal production (CAC, 2018)

Besides, due to their growth promoting properties, antimicrobials are regularly used at very low/sub-level doses as **growth promoting factor** via animal feed additives. Through, modification of intestinal microbiota composition, by reducing microorganisms which compute for nutrients and via disease preventive effects, they accelerate body gain and growth (Dibner and Richards, 2005).

Poultry Meat Production and Antimicrobial Residues

Poultry meat production is increasing worldwide with an astonishing performance by 5% since 1970 to date and currently with three-fold increment in per-capita consumption. The consumption data compiled so far showed a global increase for chicken meat demand from11 kg in 2000 to 14.4 kg per person in 2011 (FAO, 2012) with an estimate of eviscerated or ready-to-cook meat forecasted to reach 17.2 kg per person in 2030 (Terry, 2015). The increase in consumption are primarily associated with escalating population growth, urbanization and improvements in income (Delgado 2005), low chicken meat prices relative to the red meat and dietary preferences (FAO, 2017).

Increase in the demand for poultry meat puts producers under continuous pressure to produce poultry in the shortest production period possible with maximum output. This improvements in production and productivity were attained via genetic selection, advancement in feed formulation, good health management practices and the use of antimicrobials for prevention of diseases and growth promotion as feed additives (Apata, 2009). Specifically, antimicrobials mainly used in the commercial poultry production for prophylactic intervention to prevent diseases, metaphylactic use to control diseases in chickens which have been in close contact with diseased ones kept in intensive production. Antimicrobials are also used to counteract adverse consequences of stressful situation, cover faulty management conditions and for growth promotion (Dibner and Richards, 2005). Hence, treatment with antimicrobials may leave residues of the active substance or their products in the tissues and food derived from poultry meat and end up entering to the human food chain (Wassenaar, 2005). Much higher

residue levels may appear due to extra-label use, failure to comply with withdrawal periods, wrong route of administration and poor production practice (Jeong et al., 2010 and AVMA, 2015).

Occurrence of Antimicrobial Residues

More recently, usage of antimicrobials, for metaphylactic, growth promotion and feed conversion efficiency improvement purposes at normal, sub therapeutic and higher doses is becoming common. These without prescription and prolonged time use of antimicrobials will lead to the occurrence of residues in food derived from tissues of treated animals. Moreover, inappropriate prescription of antimicrobials for the treatment of viral infection (which are not responsive to antimicrobials) and use of antimicrobials to prevent secondary bacterial infections are more aggravating residue occurrence in animal originated foodstuff. Failure to keep instructions for antibiotic use (not abiding with withdrawal period), poor treatment record keeping, and problem to properly pinpoint treated animals could also lead antimicrobial residues to enter in to the food derived from animals treated with these antimicrobial drugs (Draisci, 2001; Darwish et al., 2013).

Currently Emerging Outlooks and Antimicrobials Use in Farmed Animals

Antimicrobials should be used in food-producing farmed animals when their use will result in better animal health and welfare (AVA, 2017; OIE, 2018) in responsible and prudent way. In addition, it should be known that, the use of such chemicals for growth promotion with no risk assessment is not responsible and judicious ways to use them. The use of antimicrobial agents in food producing animals causes residues in animal source food. Sequentially, the presence of these residues in animal originated foodstuffs might cause various potential public health risks and play a role to the spread of antimicrobial resistance (AMR) across the food chain (Lee et al., 2001; Okocha et al., 2018). AMR is becoming public and animal health concern of the globe, influenced by both human and non-human antimicrobials (OIE, 2018).

As per the criteria set by OIE, antimicrobial agents classified in to three, veterinary critically important antimicrobials (VCIA), veterinary highly important antimicrobial agents (VHIA) and veterinary important antimicrobial agents (VIA) (Table 2). If the importance of the antimicrobial agent is responded 'yes' by more than 50% of OIE member countries and if it is identified as essential drug for the treatment of specific infections with lack of sufficient therapeutic alternatives then the agent is regarded as a **VCIA**. Either if the drug is required by more than 50% of OIE member countries or if it is an essentially identified drug for the treatment of specific infections, it is a **VHIA**. If the compound is found in state countries where it is required and if not the only essential drug for the treatment of a specific disease with no shortage for sufficient alternative therapy, then the drug considered as **VIA** (OIE, 2018; EMA, 2019).

Fluoroquinolones, 3rd 4th generation of Cephalosporin and Colistin are from the VCIA list are considered to be critically important drugs both for human and animal health like, (Table 2) (OIE, 2018). Therefore such types of 'third line antimicrobial' agents (Table 2) have not to be used as preventive treatment via feed in the animal(s) to be treated. Such antimicrobial agents not to be used as a first line treatment unless justified, when used as a second line treatment, they should ideally be based on bacteriological sensitivity test results. Off label, use should be limited in cases where no choices and in agreement with the national legislation and their use as growth promoters should be prohibited urgently (AVA, 2017, OIE, 2018). Third line antimicrobials should be used as a last alternative when there are no other drug options authorized for the respective target bacteria and indications, which possible only after susceptibility testing has been completed (AVA, 2017). Therefore using such kinds of antimicrobials needs considerations of currently emerging outlooks from AMR and other residue related public health risks perspectives. From public health viewpoint, antimicrobials residue in food of animal origin, may pose a health risk for instance on some individuals like the occurrence of allergic reaction, the transfer of resistant bacterial genes and others might be carcinogenic or fatal, hence their use have been prohibited by the Food and Drug Administration (FDA, 2019).

Table 2.
Veterinary Important Antimicrobial Agents Commonly Used in Food Producing Animals

Class (Subclass) of Antimicrobials	**Commonly used food producing animals**		**Remarks (References)**
	Cattle	**Poultry/Avian**	
Aminoglycosides:	Streptomycin*, DH-Strep. Gentamicin^, Kanamycin* Neomycin*, Apramycin[@2]	Neomycin* Apramycin[@2] Spectinomycin[@2]	VCIA (OIE, 2018) ^ (AVA, 2017)
Amphenicols :	Chloramphenicol^ Thiamphenicol* Florfenicol*	Chloramphenicol^, Thiamphenicol*, Florfenicol*	^(CAC, 2018) ^(AVA, 2017) VCIA (OIE, 2018)
Beta-lactams (β-lactamase sensitive and resistance):	Amoxicillin*, penicillin* Ampicillin*, Cloxacillin[@2] Amoxicillin+clavulanate[@2]	Amoxicillin* (not layers) Ampicillin* Amoxicillin+clavulanate[@2]	VCIA (OIE, 2018) * &[@2] (AVA, 2017)
Cephalosporins:			
Cephalosporins 2nd	Cefuroxime[@2]	-- (not used)	VHIA (OIE, 2018)
Cephalosporins 3rd	Ceftiofur[#3], Ceftriaxone[#3]	Ceftriaxone[#3]	VCIA (OIE, 2018)
Cephalosporins 4th	Cefquinome[#3]	--	
Lincomycin	Lincomycin[@2]	Lincomycin[@2]	VHIA (OIE, 2018)
Macrolides	Erythromycin*, Spiramycin* Tulathromycin[@2], Tylosin*	Erythromycin* (not layers), Tylosin* (not layers)	VCIA (OIE, 2018)
Nitrofurans	Banned/Prohibited^	Banned/Prohibited^	^(CAC, 2018)
Quinolones: /Fluoroquinolones :	Flumequin, Oxolinic acid, Ciprofloxacin[#3], Difloxacine Enrofloxacin[#3]/	Flumequin, Oxolinic acid, Ciprofloxacin[#3] Enrofloxacin[#3]	VHIA (OIE, 2018) VCIA (OIE, 2018)
Pleuromutilins:	---	Tiamulin[@2]	VHIA (OIE, 2018)
Polypeptides (Polymyxins Colistin)	Polypeptides cyclic: Polymyxin B[#3] Colistin	-- Colistin	#3 (AVA, 2017) VHIA (OIE, 2018)
Sulfonamides:	Sulfonamides* Trimethoprim+ sulfonamide[@2]	Sulfonamides* Trimethoprim+sulfonamid[@2]	VCIA (OIE, 2018) VCIA (OIE, 2018)

Tetracyclines:	Chlortetracycline* Doxycycline* Tetracycline* Oxytetracycline*	Chlortetracycline* Oxytetracycline*(not layers), Doxycycline*	VCIA (OIE, 2018)

∗= First line antimicrobials, @2= Second line use #3 third line antimicrobials, ^ = Prohibited/banned drug from use in food animal production (e.g. Nitrofurans, Gentamicin, Chloramphenicol); AVA= Australian veterinary association; CAC= Codex AC, 2018 and OIE, 2018)

Safety Evaluation of Veterinary Antimicrobial Residues

The approach to safety evaluation of residues of VMPs (antimicrobials) within EU under committee for medicinal products for veterinary use (CVMP) is similar to that employed by the joint FAO/WHO expert committee on food additives (JECFA) of CAC (JECFA, 1997; 2013). In general, safety evaluation of VMPs must consider not only the toxicological properties but also their pharmacological properties. The other point is, the residue to which consumers of ASF are exposed may not necessarily be the same as the parent drug substance, meanwhile, the parent molecule may be extensively metabolized within the treated animal. After the completion of the various pharmacological, toxicological and other tests, to determine the safety of the substance, the first step in safety evaluation is establishment of (ADI) (CVMP, 2005).

Acceptable Daily Intake (ADI). ADI is an estimate of the amount of a veterinary drug in food that can be ingested daily over a lifetime without appreciable health risk to the consumer. It is the safe concentration that can be expressed on body weight bases (µg/kg or mg/kg) (CVMP, 2005). The ADI calculation is based on the array of toxicological safety evaluation and it can be derived from sub-acute, acute and long-term or chronic exposure studies to the drug and its potential impact (FAO/WHO, 2009; Alan et al., 2017).

Maximum Residue Limit (MRL). MRL is "the maximum concentration of residue, resulting from the use of a VMP, which may be recognized/permitted as acceptable/safe in a food". MRLs of approved veterinary drugs in food are set with legally permitted quantities of parent drugs and/or metabolites in food products of treated animals that are safe for consumers (EC/EU, 2009). This establishment of MRL represents one of the several standard options for risk managers to limit the presence of

unwanted substances. In the EU, for setting limits in safety enhancement, the major action was the introduction of requirements for MRLs of veterinary drug residues in food of animal origin. Even if various activities have been made to harmonize MRLs worldwide, through the support of World Trade Organization (WTO) and CAC, MRLs are still differ from one country to another depending on the local food safety regulatory agencies and drug usage patterns (Table 3). Besides most developing countries have yet to develop their own MRLs (EC/EU, 2010; Anadon et al., 2012).

$$\textbf{Safe concentration} = \frac{(\text{ADI}) \times (\text{Body weight})}{\text{Food consumption factor}}$$

Concerns Over |Veterinary Antimicrobial Residues in Ethiopia

Antimicrobial residues are spreading swiftly, regardless of geographical, economical, or legal differences between countries (Darwish et al., 2013). Because of this, the concerns over food residues are becoming more economic as well as public health related. In Ethiopia, concerns demonstrated over a decade about the presence of antimicrobial residues, in the meat and milk supplies. A research conducted between October 2007 and May 2008 in Debre Zeit dairy farms from milk, indicate 8.5% antibiotic residue prevalence. The antibiotic residue positive samples, which showed residues of oxytetracycline and penicillin G 70.58% and 20.58%, were above the WTO/FAO/CAC established MRL of 100µg/l and 4µg/l respectively (Desalegne, 2010). Also in another study conducted from October 2006 to May 2007 on tetracycline residue levels in beef at Addis Ababa, Debre Zeit and Nazareth slaughterhouses showed oxytetracycline residue 71.3% out of which 48% of the edible tissues had residue levels above the recommended MRL (Addisalem and Bayleyegn, 2012). Another latest study done on ‘antimicrobial residue occurrence and its public health risk on beef meat in Debre-Tabor and Bahir-Dar’, cities found in the northwest part of Ethiopia, showed the occurrence of 43.6% positive results for oxytetracycline (Agmas and Adugna, 2018).

Table 3.

Regulatory MRL values for selected antimicrobial in animal originated food

Group of Antimicrobial	**Pharmacologically active substance (Remarks)**	**Target tissue (Cattle)**	**Maximum Residue limit (MRL, µg/kg)**		**ADI & ARfD (µg/kg bw) (References)**
			EU	**^CAC**	
Aminoglycosides	Streptomycin	Muscle	50	100	ADI= 0-20
		Liver	200	2000	ARfD= --
		Kidney	750	5000	(CAC, 2015; 2018)
		Milk(µg/L)	100	200	(EC/EU, 2010)
Amphenicols	Chloramphenicol	Muscle	-	-	Prohibited/No safe
		Milk(µg/L)	-	-	level (CAC, 2018)
Beta-lactams	#Amoxicillin	Muscle	50	50	ADI= 0-0.07
		Liver	50	50	ARfD= 5 based on
		Kidney	50	50	microbiological
		Milk(µg/L)	4	4	effect (CAC, 2018)
Macrolides	Erythromycin	Muscle	200	100	ADI= 0-0.7 and
	(in Chicken)	Liver	200	100	(CAC, 2015; 2018)
		Kidney	200	100	^All food
		Eggs	150	50	producing species
Nitrofurans*	Furazolidone,	Muscle	-	-	**Prohibited**
	Nitrofural	Milk(µg/L)	-	-	(EC/EU, 2010)
					(No safe level)
Sulfonamides	Sulfadimidine	Muscle	100	100	ADI= 0-50 µg/kg
	(all substances	Liver	100	100	(CAC, 2018)
	belonging to the	Kidney	100	100	(EC/EU, 2010)
	sulfonamide group)	Milk(µg/L)	100	50	
Tetracyclines	Chlortetracycline,	Muscle	100	200	ADI= 0-30 µg/kg

	Doxycycline,	Liver	300	600	(CAC, 2018)
	Oxytetracycline,	Kidney	600	1200	
	and Tetracycline	Milk(µg/L)	100	100	

Antimicrobials Residue Detection and Quantification Methods

Screening Methods of Analysis

Microbiological Techniques (Microbiological Assays). The microbiological methods used for detecting antimicrobial residues in food of animal origin are based on inhibiting microbial growth, microbial receptor activity and enzymatic reactions and could be applied to all types of matrices, usually milk, meat, eggs and honey. Microbial inhibition assays involve culturing a microorganism from a standard strain, usually Bacillusstearothermophilus, Bacillus subtilis, Bacillus cereus, Micrococcus luteus, Escherichia coli, Bacillusmegatherium, Sarcinaluteaand Streptococcus thermophiles (Neaves 1999).

Immunochemical Techniques. The immunological methods are based on the interaction antigen–antibody which is very specific for a particular residue. Immunochemical methods represent an important tool for determining drug residues, given their high specificity, they lead to analytes being determined in samples having had very reduced prior cleaning treatment. These assays are based on the reaction of an antigen binding to a specific primary antibody or for each antigen, analogously to an enzyme substrate reaction. The most common immunochemical methods include the enzyme-linked immune sorbent assay (ELISA), direct and indirect competitive enzyme linked immunosorbent assays, immune affinity chromatography (IAC), radioimmunoassay (RIA), the enzyme-monitored immunotest (EMIT), the fluorescent immunoassay (FIA) and the chemiluminescence immunoassay (Roda, 2003).

Confirmatory Method of Analysis /Physicochemical Techniques

Physicochemical methods are mainly used for isolating, separating, quantifying and confirming the presence of veterinary drugs residues in the samples of edible products (Aertset al, 1995). Separation methods are founded on the principles of chromatography and are generally coupled to high sensitivity and selectivity detection

techniques leading to quantifying compounds of interest with a high level of precision and exactitude and its clear identification at very low concentration levels. The chromatographic methods used for screening and quantification of analytes in complex matrices would be gas chromatography (GC), high performance liquid chromatography (HPLC) coupled with different detectors (UV, DAD and FLD), liquid chromatography coupled with mass spectrometry (LC-MS), Spectrophotometric methods are also used either alone or coupled to chromatographic or immunochemical methods (Reig, 2006).

High Performance Liquid Chromatography(HPLC). HPLC is getting expanded use in quality control laboratories because of the advantage to analyze concurrently multiple residues in a sample in relatively short time. Recent developments of high speed HPLC can reduce sample treatment and analysis time. In addition, it is state of the art and computer-controlled, which enable as a screening technique of residue analysis. The next step after initial screening with HPLC is the injection of the presumed positive samples in a system combining HPLC with triple quad mass spectrometry detection. In this sense, the coupling of HPLC with MS/MS can substantively reduce the analysis time. The use of HPLC-electrospray ionization (ESI) tandem mass (two mass analyzers separated with a collision cell) spectrometry has been proposed as a simultaneous screening-confirmatory technique (Puente, 2004).

CHAPTER III
MATERIALS AND METHODS

The study was conducted using analytical grade chemicals, reagents, certified reference materials (CRMs), laboratory grade equipment and glassware. Basic instruments used in this research were calibrated and their proper functioning checked before use. The art of state laboratory instruments were properly installed and there installation and performance qualifications have been confirmed in advance.

Analytical Standards, Chemicals and Reagents

Certified reference standards of the highest purity grades from four families of antimicrobials with known available potency such as penicilin-G (100%), enrofloxacin (99.2%), sulfadiazine (99.7%), sulfadimidine (100%), oxytetracycline (91.3%), tetracycline hydrocloride (97.5%), and doxycycline hyclate (85.7) were used purchased from United States Pharmacopoeia (USP) (USP, 12601 Twinbrook Pkwy, Rockville, MD, +1-301-881-0666) (Annex A to C).

Chemicals such as ACN (acetonitrile) and MeOH (methanol), HPLC grade were supplied from Sisco Research laboratories (SRL) (Maharashtra, India). Disodium EDTA dihydrate (99%), anhydrous dibasic sodium phosphate ($Na_2HPO_4.2H_2O$, 99%), anhydrous citric acid (99%), formic acid (FA) (99%) was obtained from (Val de Reuil, France). Deionized water of resistivity 18.2 MΩ cm^{-1} was produced in-house using Barnstead GenPure Pro UV – TOC/UF water purification system from Thermo scientific (Langenselbold, Germany). Sample extraction cartridges were provided by Waters (Milford, Massachusetts, USA) PRiME Oasis Hydrophilic-Lipophilic Balance (HLB) cartridges (6cc, 200 mg and sorbent type Oasis® HLB, 30µm, PN= 186008057).

Equipment and Instrumentation

In the study, we have used different types of sample processing apparatus and state-of-the-art laboratory equipment for analytical activities. The main instruments which, were used in sample preparations include, analytical microbalance (± 0.01mg, Sartorius Lab instruments Goettingen, Germany), pH meter (HANNA pH-ORP, HI11310; USA). Sample homogenization, mixing, extraction and related activities were done using meat blender, vortex mixer (Bio Cote Sturt, UK), model 75-wrist-action shaker (Burrell Scientific, USA), refrigerated centrifuge (HERMLE, Z446K; Germany). While sample cleanup and concentration steps were arrayed using vacuum manifold (Supelco, Germany) and nitrogen sample concentrator (MultiVap 54 Lab Tech, USA) respectively.

Besides, other apparatuses like micro pippet and tips of (10, 200, 1000 µl), Duran bottles, bottle top volumetric dispensers (BrandTech scientific, Inc., Germany), beakers, different volumes of 'class A' volumetric flasks and graduated measuring cylinders, screw caps centrifuge tubes/Falcon tubes (15 mL and 50 mL), spatulas and funnels. All glassware and other apparatuses were kept cleaned and dried in a drying cabinet.

Determination of analytes of interest was performed with an Agilent 1290 Infinity II Ultra-High Performance Liquid Chromatography (UHPLC) system. It was equipped with a reversed phase (RP) Phenomenex® Synergi hydro- (4.6mm × 150 mm; 4 µm) analytical column through security guard cartridge system (4x3.0 mm) for chromatographic separation. The LC system was coupled with an Agilent 6470 LC/TQ/ triple quad mass spectrometer (Agilent Technologies Ltd., Singapore) via an electrospray ionization source which was operating with MassHunter software.

Research Design

A cross-sectional study was carried out to know the occurrence of antimicrobial residues in raw beef and eviscerated poultry meat samples in the study area. Seven veterinary drugs (Penicillin G, Enrofloxacin, Sulfadiazine, Sulfadimidine Doxycycline,

Oxytetracycline and Tetracycline) were selected from four classes of veterinary antimicrobials. The antibiotics specifically selected based on previously conducted research reports on assessment of prudent use of VMPs in Ethiopia (Beyene et al., 2015; Gemeda et al., 2020).

The study was conducted on raw beef and eviscerated poultry muscle meat samples collected from three sub cities of Addis Ababa ('Akaki Kality', 'Nifasilk Lafto' and 'Kolfe Keraniyo) and Bishoftu town, central Ethiopia. Addis Ababa is capital city of Ethiopia and the seat for federal government of the country, African Union head quarter, UN-ECA (United Nation Economic Commission for Africa), presidency of Oromia regional state and different embassies and diplomatic offices. Bishoftu is positioned 45 km South East of Addis Ababa at 9^0N latitude, 40^0E longitude and at an altitude of 1850 meters above sea level (Zeleke et al., 2005) (Shawu et al., 2019).

Sampling Techniques and Sample preparations

Sampling Techniques and Samples

Available data related to antimicrobial residues in the study area is very scant. However, to estimate sample size, previous research reported by Bedada and Zewde (2012) (48% positive sample result) was considered. Consequently, the choice of a 95% confidence interval (z=1.96) and a 10% absolute precession, a minimum sample size estimate of 96 was obtained using the formula (Thrusfield *et al.,* 2018) given below. To increase validity of the sample size, the obtained sample number as per the formula was almost doubled and 180 beef meat samples were collected.

$$Sample\ size\ (n) = \frac{p\ (1-p)z^2}{d^2} \qquad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

Accordingly, a total of 180 beef muscle meat samples were collected from October /2020 to January /2021 and 120 eviscerated poultry meat samples were collected and analysed from February 2021 to the middle of June/2021. About 500 g fresh beef muscle tissue (CAC, 2009) and the whole eviscerated poultry meat samples were purchased purposively from butcheries and supermarkets in Addis Ababa city and Bishoftu town. Each sample was collected using a sterile sample collection

polyethylene/plastic bag individually identified and properly labeled using labeling tape. Names, dates, places and retail outlets (Butcher's shops and Supermarkets) of sample collection were recorded with corresponding codes simultaneously. Individually collected and packed samples placed in an icebox during shipping to Animal Products quality, safety and residues testing laboratory of Ethiopian Agriculture Authority, the cold chain being maintained. After arrival at the laboratory center, apparent fats from each beef muscles and skins from chickens sample were removed away, minced and homogenized using meat blender. From each homogenized beef and poultry meat samples, 4.0 g was accurately weighed, in duplicates, in a 50 ml falcon tubes and kept frozen (≤ 20 °C) until the time of samples extraction and clean up.

Preparations of Solutions

Mobile phase A or Aqueous mobile phase (Water with 0.1% FA): 1.0 mL of FA added to a half-filled volumetric flask of 1.0-liter capacity, then brought to volume with deionized water. The flask was degassed offline using Sonicator for 10 minutes, and then transferred to the aqueous reservoir of the machine. Then Mobile phase B or organic mobile phase (ACN with 0.1% FA) was prepared by mixing 1.0 mL of FA pipetted into a 1 L volumetric flask and brought to the volume using ACN. The mobile phase degassed and transferred to organic mobile phase reservoirs.

Next to the mobile phase, extraction solution (Acidified ACN + McIlvaine Buffer + 0.1M Na_2EDTA) was prepared as follows:- 0.1% FA in acetonitrile: About 0.40 ml of formic acid was pipetted and mixed with 400 mL of acetonitrile in a graduated cylinder and then transferred to a dispenser bottle for storage.

McIlvaine buffer (mixed citrate-phosphate): 14.21 g anhydrous dibasic sodium phosphate and 9.605g anhydrous citric acid were separately dissolved well each in 500 ml de-ionized water. Then 308 ml citric acid solution (0.1M) and 192 mL phosphate solution (0.2M) were mixed carefully in Duran bottle (pH was maintained at 4.00 ± 0.05). McIlvaine Buffer/0.1 M Na_2EDTA: 18.61 g disodium EDTA dihydrate added in 500mL McIlvaine buffer and sonicated. Eventually, the diluent was prepared by mixing

80 mL of deionized water and 20 mL of acetonitrile were measured using graduated cylinders and combined in a 100 ml flask.

Preparation of Standard Solution

The stock solutions were prepared at concentrations corresponding to 1.0 mg/mL (1000µg/ml) taking in to account stability and solubility of the drug in the solvent. Standard solutions were prepared separately by transferring 10.0 mg equivalent of the base materials quantitatively in to a 10.0 ml volumetric flasks. Sulfadiazine, enrofloxacin and three of the tetracyclines were dissolved in methanol and penicillin-G was prepared in deionized water and diluted to the volume accordingly.

Intermediate standard solutions for sulfadiazine, enrofloxacin and the tetracyclines were prepared by pipetting 400µl aliquot of stock and diluting in a 10.0ml volumetric flask with methanol to 40ng/µl. Intermediate standard solution for penicillin G (20 ng/µl) was prepared by transferring 200µl stock and diluting to 10 ml final volume with water. When they are not in use, all the stock and intermediate standards were stored in amber vials at ≤ -20 ^{0}C. A working standard (WS) was made by pipetting 1.0 ml of intermediate solutions into a 10 ml volumetric flask and diluting to the mark with diluent (80:20 water/Acetonitrile) giving final concentrations of 4 ng/µl and 2 ng/µl respectively (Table 4).

Table 4**.**

Spiking Procedure and Volume and Target Tissue Concentrations

SDZ, SDM, ENR and TTCs		**PnG**		**SDZ, ENR TTCs (4ng/µL)**	**PnG (2ng/µl)**
Spiked Level	**Spiked volume (µl)**	**Spiked Level**	**Spiked volume (µl)**	**Concentration in 4g muscle tissue (**µg/kg)	
0.5xMRL	50	0.5xMRL	25	50	25
1 x MRL	100	1 x MRL	50	100	50
1.5xMRL	150	1.5xMRL	75	150	75

2xMRL	200	2xMRL	100	200	100
2.5xMRL	250	2.5xMRL	125	250	125
3xMRL	300	3xMRL	150	300	150

Sample Preparations

Blank Samples and Internal Quality Control Samples Preparation

For the method validation purpose, blank samples and meat samples fortified with antimicrobial compounds of interest, at six working ranges were prepared as internal quality control samples/matrix matched calibrants. Hence, three batches of matrix-matched calibrants were prepared over three different days (Day 1, Day 2 and Day 3). Each batch consisted of 21 samples fortified with the six antimicrobials (SDZ, SDM, OTC, TTC, ENR, DXY and PnG) at, 0.5, 1, 1.5, 2, 2.5 and 3 times MRLs concentrations and 3 blank samples all being prepared in triplicates. Based on the MRLs of each antimicrobial residues, the concentrations were 50, 100, 150, 200, 250, 300 µg/kg and for PnG 25, 50, 75, 100, 125 and 150 µg/kg. Along with, for determination of recovery and precision, nine (9) spiked samples each in triplicates were prepared by spiking blank samples at 0.5, 1.0 and 1.5 times concentrations of the MRLs. Afterward, all the validation samples were kept for a period of 30 minutes in a dark place to allow equilibration of the spiked antimicrobial standards with the meat matrix before starting the extraction step. Together with each batch of validation samples, two quality control samples were prepared, matrix-matched reference standard fortified at MRLs post spiked on the matrix (after sample underwent all preparative steps) and a reagent blank, which doesn't contain the matrix or any analytes of interest in order to eliminate false-positive and ensure that the system is under control.

Solid Phase Sample Extraction and Clean up Procedures

In this study, the quality control samples were prepared in triplicates, whereas the test samples were prepared and analyzed in duplicates and similar extraction and clean up procedures/steps were employed for experimental and test samples. The frozen ground fortified and blank meat samples were, taken out of the deep freezer and, allowed to thaw overnight at 4°C, until the subsequent sample preparations steps. The

quality control samples were spiked with appropriate volumes of working standards at this stage (Table 4).

Ten milliliter of the extraction solution (2 ml Na_2EDTA-McIlvaine buffer and 8 ml of acidified ACN with 0.1% FA were added using a calibrated solvent dispenser in sequence to the falcon tubes. Then the tubes capped tightly and vortex mixed briefly for 30 seconds and allowed to stand for 30 min in a dark. Subsequently, the sample mixtures were shaken vigorously for 15 minutes using a wrist-action mechanical shaker. After shaking, the sample tubes were centrifuged for 15 minutes at 4500 rpm at 4°C. Then samples purified by Solid Phase Extraction (SPE) technique using 12 ports SPE vacuum manifold (Supelco, Germany). After carefully mounting Oasis PRiME HLB cartridges on vacuum manifold, the supernatant was loaded from the 50 ml falcon tube via oasis PRiME HLB cartridges. The process does not required cartridge conditioning and was not performed (He et al,. 2017). Then the eluted solutions were directly collected in to 15 ml scaled conical plastic centrifuged tubes. About 5ml of the clean extracts collected in to another sample tubes and evaporated at 40 ^{0}c under a gentle stream of nitrogen gas nearly to dryness (0.10 ml) using MultiVap 54 Lab Tech a nitrogen gas streamed sample concentrator with a half filled water bath. The sample concentrator was coupled with an online nitrogen gas generator (Annex H).

Afterwards, the concentrated residues were reconstituted with 1 ml initial mobile phase, recapped and vortexed for 30 seconds, centrifuged for 15' at 4500 RPM at 4 ^{0}c. Finally, very clear supernatant supposed to contain antimicrobial residues of interest, was transferred into autosampler vials and closed tightly to make it ready for injection. Finally, 10 µl injection volume was injected in to the UHPLC-MS/MS system.

LC-MS/MS Method of Analysis

The analysis was performed by UHPLC of an Agilent 1290 Infinity II system (Agilent Technologies Ltd., USA) interfaced to an Agilent 6470 LC/TQ/ triple-quadrupole mass spectrometer (MS/MS) equipped with Agilent jet stream electrospray

ionization source, which was operated in positive mode (AJS-ESI +) and controlled by MassHunter software.

Antimicrobials separation was chromatographically achieved on Phenomenex Synergi hydro-RP, (4.6 mm × 150 mm; 4 µm, 80 A° dimensions) column with guard cartridge system (4x3.0 mm). The mobile phase was a binary gradient mobile phase with flow rate, which was set at 1.0 ml/min for a total run time of 17 min (Table 5). Methanol with 0.1 % FA (Mobile phase-A) and acetonitrile with 0.1% (v/v) (Mobile phase-B) were used.

Liquid Chromatography mass spectrometer condition (LC-MS/MS)

The column compartment was operated at 30 °C, while the auto-sampler temperature was set at 10 °C. The injection volume was 10 µL. The auto sampler was rinsed after each injection using a solution of H_2O:MeOH (50:50, v/v). The system was conditioned with a mobile phase for more than an hour prior to actual analysis. In this study, gradient elution of mobile phase was used for separation of multi-class antimicrobials.

Table 5.

Mobile Phase Gradient Profile

S. No.	Time (min)	Mobile phase -A (%)	Mobile phase -B (%)
1.	0.00	90	10
2.	4.50	90	10
3.	4.60	80	20
4.	10.50	80	20
5.	12.00	20	80
6.	15.00	20	80
7.	17.00	90	10

The Electrospray ion source in +ve modes specific to Agilent company (AJS-ESI +) was used with data acquisition in multiple reaction monitoring (MRM) mode and analyzed

using MassHunter software. The triple quad mass spectrometer parameters were adjusted and the source parameters were set as follows: gas temperature, 350 °C; gas flow rate, 12 l/min; sheath gas temperature, 250 °C; sheath gas flow, 11 L/min; nebulizer pressure, 40 psi; capillary voltage, 4 KV; nozzle voltage, 500 V.

Method Validation

The analytical method was validated and the obtained results were quantitatively confirmed and interpreted based on European Union Commission Decision (CD) 2002/657/EC, EU 2021/808 and Codex Alimentarius Commission (CAC) CAC/GL 71-2014 guidelines (C.D, 2002; CAC, 2014 and EC, 2021). Method performance parameters including specificity, matrix effect (ME), linearity, accuracy (recovery), and precision (repeatability and reproducibility, limit of detection (LOD), limit of quantitation (LOQ), decision limit (CCα) and detection capability (CCβ) were determined for each analyte included in the investigation to validate requirements of the analytical procedure.

System Suitability Check

System-suitability test was performed on daily bases using a mixed antimicrobial standard solution of five replicate injections prepared at the concentration of MRLs. The performance was checked before starting the actual sample analysis activity and the required parameters were evaluated.

Compound Identification

The confirmatory identification of authorized substances using the LC-MS/MS technique was done by obtaining two mass transitions at the same retention time (RT). RT of the analytes in the chromatogram of sample solution should correspond to that of calibration solutions of standards within ± 2.5 % deviation in RT (C.D., 2002; EC, 2021) and ± 0.1 min absolute deviation according to the SANTE/12682/2019 guidelines. However, the EU guideline stipulates the use of internal standards in the calculation of the relative retention time deviation, and therefore the SANTE guideline was followed instead. The relative intensities or ion ratio of the diagnostic ions are expressed as a

percentage of the intensity of the most abundant ion and the acceptance criteria for ion ratio, not more than 25% (± 25%) was used (C.D, 2002; CAC, 2014 and EC, 2021)

Specificity and Matrix effect

In order to verify the method specificity and confirm the absence of potential interfering compounds around the retention time of each antimicrobials, 20 blank extract of the matrix and 20 fortified/spiked extracts were analyzed. Then checked for the presence of significantly interfering peak at each mass transition of targeted antimicrobials within 2.5 % margin of the retention time.

Relative matrix effect. Determination of relative matrix effect was done using 20 blank samples which were fortified with mixed standards (matrix matched standard = MMS) at MRL level and 20 solvent matched standards (Standard dissolved in a solvent) analysed together. Relative matrix effect or matrix factor (MF) (EC, 2021):

$$MF = \frac{\text{Peak area of MMS}}{\text{Peak area of solution standard}} \quad (2)$$

Linearity

Linearity of the method was evaluated by constructing a matrix-matched calibration curves (MMC) of aliquots obtained from samples spiked with antimicrobial standards of interest. Calibration standards were prepared by spiking beef matrices with a known quantity of target analytes of calibration range comprising the MRL. The spiked concentrations were 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 times of the corresponding MRLs, each in triplicates, with expected concentration equidistantly ranging from 25 to 300 µg/kg. The plot of the MMC for each compound was based on the peak areas of each analyte at various concentrations.

Accuracy

The accuracy, expressed in terms of recovery, calculated by dividing the mean measured or calculated concentration of the analyte to respective spiked level or

expected concentration multiplied by 100, to express the result as a percentage Commission Decision 2002/657/EC or EU 2021/808 (C.D, 2002; EU, 2021).

$$\% \text{ Recovery} = \frac{\text{Measured content x 100}}{\text{Spiked or expected concentration}} \quad (3)$$

Precision

Precision of the method assessed for each analyte using aliquots of a blank matrix fortified in triplicates at concentrations corresponding 0.5, 1 and 1.5 times of the MRLs set by EU legislation (2002/657/EC, 2021/808/EU). Precision of the procedure was evaluated in terms of day-to-day repeatability (Sr) and within-laboratory reproducibility (WLR). Precision of inter-day and intra-day variation was calculated as the relative standard deviation:

$$\text{Precision} = \frac{\text{Standard deviation} \times 100}{\text{Mean}} \quad (4)$$

Methods used to support EU or Codex MRLs for veterinary drug residues should meet the performance standards for precision and accuracy listed in Table 4 (Annex), where CV refers to the coefficient of variation determined by test portions of blank matrix fortified prior to extraction.

Limits of detection and Limit of quantification

The limit of detection (LOD) is the lowest concentration of antimicrobials that can be detected at a specified level of confidence. In order to determine LOD, a concentration of 10 µg/kg (one tenth of MRLs) of matrix-matched samples of the six mixed standards were prepared in ten different replicates. Then independent measurements of each samples were taken ten times and their standard deviation (Stdv.) were calculated and the LOD determined as LOD = 3 X Stdv. In similar fashion, to know the limit of quantification (LOQ), “the lowest concentration at which the performance of a method or measurement system is acceptable for a specified use”, calculated as LOQ = 10 X Stdv. (Magnusson and Örnemark, 2014).

Decision limit, CCα and Detection capability, CCβ

The decision limit (CCα) is the lowest concentration level that can be detected in a sample with 5% of false positive decision (α = 5%). The detection capability is the concentration at which a method is able to detect the analyte with a statistical certainty of 1– β (β=5%). When the determined concentration is lower than CCα, the sample can be declared compliant which means analyte absent or present at a concentration lower than the MRL) with a confidence level of 95% (or 1 − α). Decisions limit and detection capabilities were determined using blank sample fortified at 0.5, 1.0 and 1.5 times MRLs, in the same experiment as accuracy and precision. The decision limit CCα (α = 5 %) and the detection capability CCβ (β= 5 %) were calculated according to the ISO 11843-1 calibration curve procedure (BSI, 2008; Verdon et al., 2006).

CHAPTER IV
Findings and Discussion

Optimization of chromatographic condition

In this particular study, screening and confirmatory method of multi drug residues analysis by UHPLC-MS/MS, designed by United States-Food Safety and Inspection Service (FSIS, 2013), was optimized for selected parameters. The chromatographic condition/column performances was optimized with respect to run time, retention time, peak shapes and resolution. The aqueous mobile phase (5% ACN, 95% water, and 0.1% FA) was modified to 100% ultra-pure water with 0.1% FA and used as gradient elution. Besides, the flow rate was adjusted from 0.5 ml/min to 1 ml/min and the operating column temperature from 40^0c to 30^0c and an excellent separation for targeted antimicrobials, SDZ, OTC, TTC, ENR, DXY and PnG with the short run time was achieved (Fig. 1).

Figure 1.
Total Ion Chromatogram (TIC) of Six Antimicrobial Standards Mix Fortified in Beef Sample Matrix

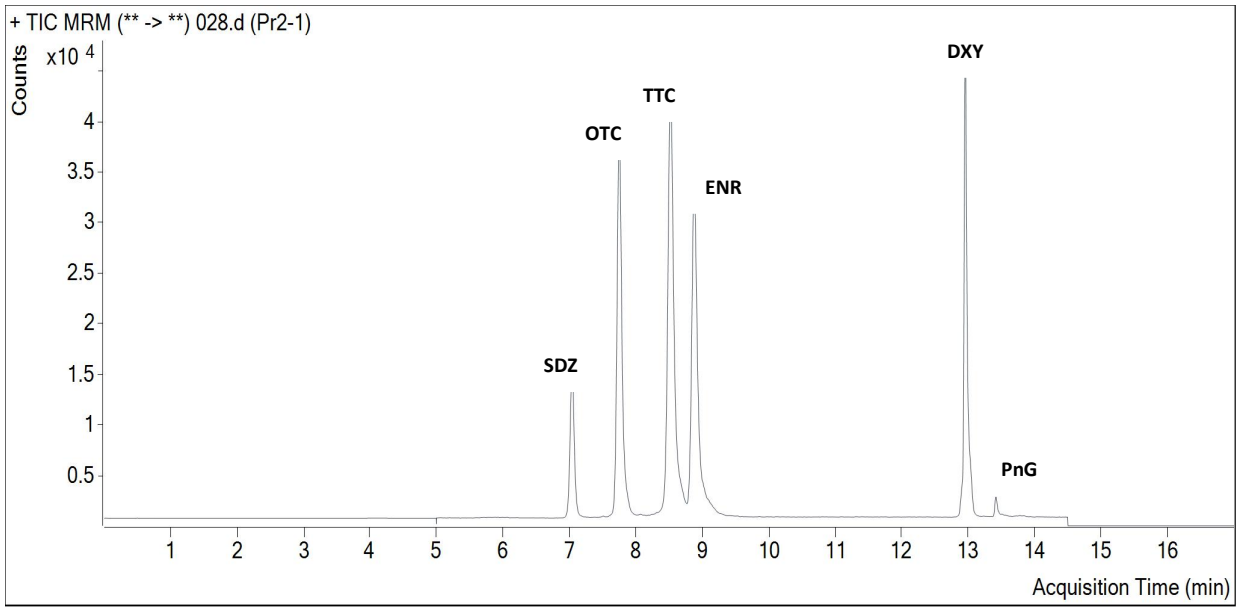
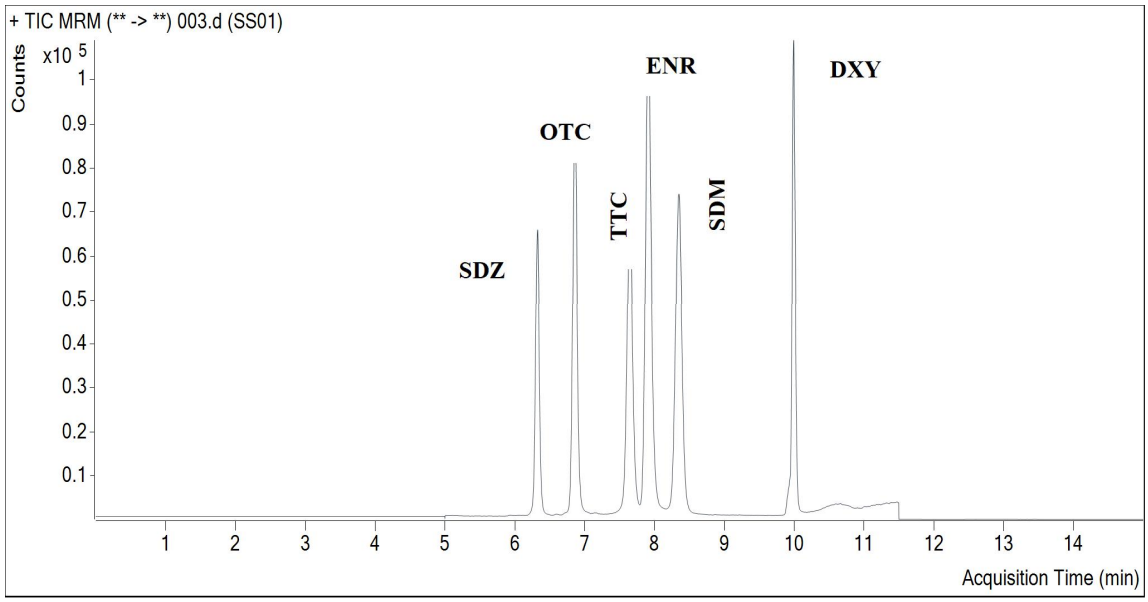


Figure 2.

Total Ion Chromatogram (TIC) of Six Antimicrobial Standards Mix Fortified in Poultry Meat Sample Matrix (SDZ, OXY, TTC, ENR, SDM, and DXY)



Accordingly, using the optimized gradient mobile phase program, it was possible to attain chromatographic separation of all the antimicrobials using RP column of 4.6

mm × 150 mm; 4 µm dimension which is similar with other researches (Mokh et al., 2020). Selecting appropriate columns and using proper composition and gradient of mobile phases are critical for obtaining optimum peak separation and reducing the ion suppression effect (He et al., 2017). For this reason, certain efforts had been devoted for choosing analytical column, appropriate mobile phase gradient composition with reasonable runtime (Fig. 1 and 2). For all the seven antimicrobials incorporated in this study, a base line peaks resolution with good width and symmetry or shape was obtained. Because of this, the selected column for this validation study was able to provide good peak resolution and shapes as shown in the figure 1 and 2 above and table 6 below.

System-suitability test result

System-suitability checking parameters such as retention time (t_R), tailing factor (T), efficiency/plate count (N), resolution (Rs) , and RSD for the peak areas with corresponding acceptance criteria set in each validation run: $T \leq 2.0$ and $N > 2000$; $Rs > 1.5$ and $RSD \leq 2.0\%$ (Thangabalan et al., 2017). The separation between the two consecutive peaks was greater than 1.5 (Ashwin et al., 2012), indicating there is no overlapping among each chromatographically separated antimicrobials peaks. All the assessed parameters were met the criteria showing suitability of flow-rate, column type, and mobile phase compositions for the chromatographic procedure as summarized in Table 6.

Table 6.
System Suitability Parameters

Antimicrobials	$\bar{t}_R \pm$ **% RSD**	$\bar{x} \pm$ % **RSD**	**Column Efficiency (N)***	**Symmetry factor**	**Resolution** (Rs)**
Sulfadiazine	7.02± 0.002	132025 ± 0.76	56083	1.06	6.279
Oxytetracycline	7.75 ± 0.0002	307648 ± 2.12	72225	1.72	5.65
Tetracycline	8.51± 0.029	639224 ± 1.49	49113	1.70	2.2

Enrofloxacin	8.86 ± 0.003	435315 ± 0.60	48803	1.83	34.87
Doxycycline	12.96 ± 0.001	377444 ± 2.01	475550	2.10	6.31
Penicillin G	13.42 ± 0.002	10567 ± 0.98	560793	2.1	6.31

$\bar{t}_R$ = Mean retention time; $\bar{x}$= mean response signal; N = Theoretical plate number

Calculations: $^{*}N = 5.54\ (t_R/w_h)^2$; $^{**}\ R_s = 1.18\ (t_{R2} - t_{R1})/(w_{h1} + w_{h2})$

Therefore, the optimized chromatographic condition of the analytical method resulted in very good separation of all the six antimicrobials belonging to the four different classes in a single run with sufficient resolution. In this study, 0.1% FA was added on both aqueous and organic mobile phase compositions to improve signal intensity of antimicrobials (Mokh et al., 2017 and Mokh et al., 2020). Acceptable peak shapes and reproducible RT were achieved with 0.1% FA added in deionized water and ACN.

UPLC-MS/MS condition

LC coupled with mass spectrometer (LC-MS/MS) technique used commonly for detection and confirmatory analysis of multi antimicrobial residues in animal source food (Lee et al., 2018, Jammoul and El Darra, 2019, Mokh et al.,2020). From the LC-MS/MS optimization, the two most intense precursor to product ion transitions per target analyte were selected to confirm positive findings for operation in multiple reaction monitoring (MRM) mode. Table 7 depicts the selected transitions and MRM optimized MS/MS parameters of all target antimicrobials in the study.

Table 7.

Multiple Reaction Monitoring (MRM) Mass Acquisition Parameters and Retention Times of Each Antimicrobials Analyzed

Classes of Antimicrobials	**Antimicrobials**	**RT (min)**	**Precursor ions $[M+H]^+$**	**Product ions [m/z]**	**Fragmentation (V)**	**CE (V)**	**CAV**
Sulfonamides	Sulfadiazine (SDZ)	7.02±0.002	251.1	108.0	96	22	7
				156.1	96	8	7
	Sulfadimidine (SDM)	8.35±0.015	279.1	186.1	120	14	7
				124.1	120	18	7
Tetracyclines (TTCs)	Oxytetracycline (OTC)	7.75±0.000	461.2	426.2	120	16	7
				443.4	120	8	7

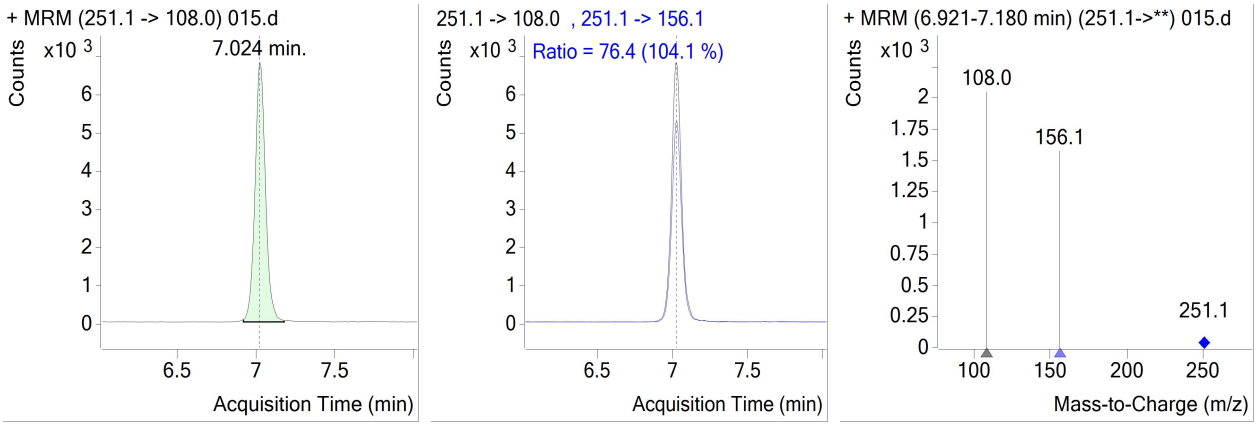
	Tetracycline (TTC)	8.51±0.030	445.2	410.2	120	16	7
				154.1	120	15	7
	Doxycycline (DXY)	12.96±0.001	445.1	428.1	120	15	7
				153.9	120	34	7
Quinolones	Enrofloxacin (ENR)	8.87±0.003	360.2	316.2	156	16	3
				342.2	156	20	3
Beta lactams	Penicillin G (PnG)	13.42±0.002	335.1	176.0	110	13	4
				160.1	110	5	4

RT, retention time; CE, collision energy; CAV, cell accelerator voltage

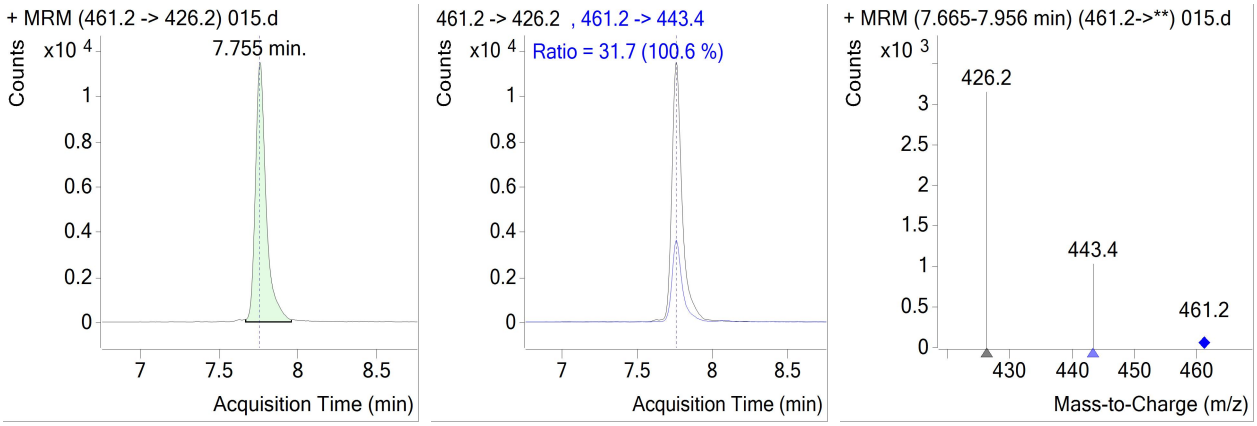
For quantification of each target antimicrobial residues, one transition of the product ion with the maximum intensity (base peak) was selected and the other used as qualifier ion for confirmation (Figure 2) according to the EU requirement (C.D. 2002/657/EC). The MRM chromatograms of the six antimicrobials belonging to four classes of veterinary drugs spiked at 50 µg in beef sample matrix were represented in Fig. 3 (A to E).

Figure 3.

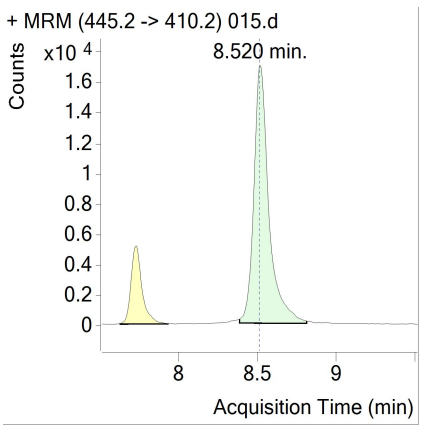
Dynamic MRM Chromatogram for selected antimicrobials, showing the transition acquisition time and mass-to-charge (m/z) ratio of abundant peak (A to F)

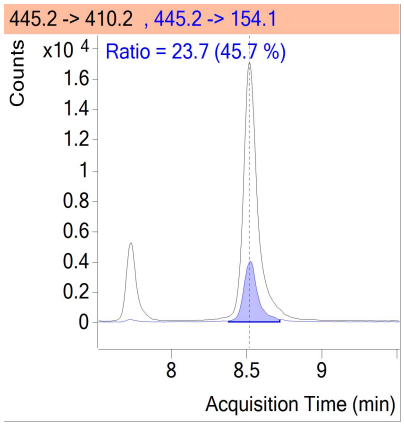


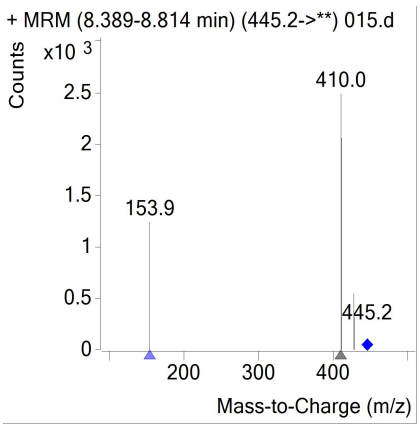
A. Sulfadiazine (SDZ)



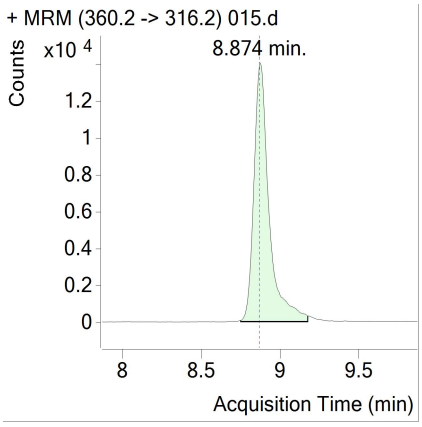
B. Oxytetracycline (OTC)

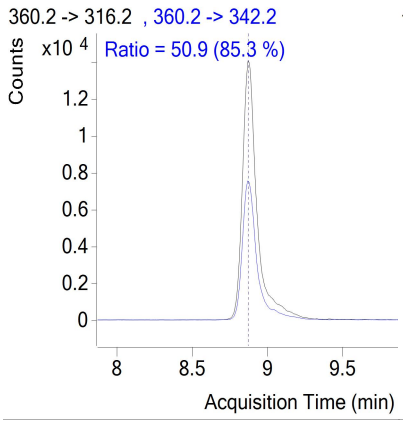


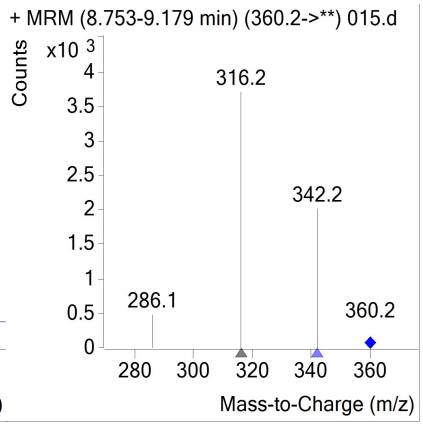




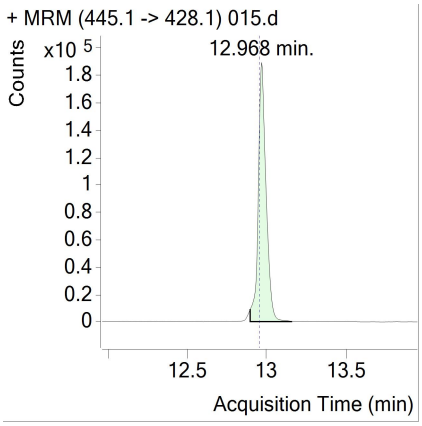
C. Tetracycline (TTC)

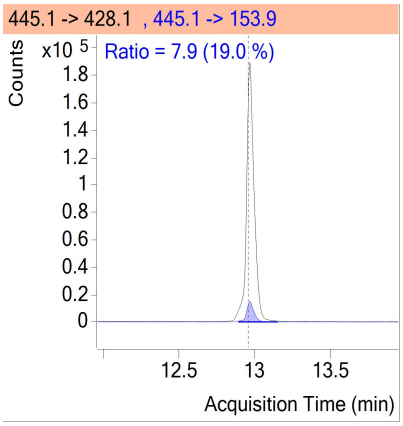


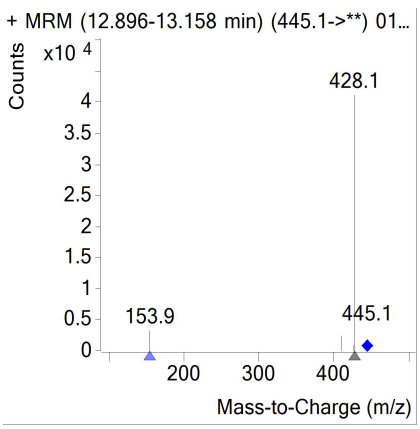




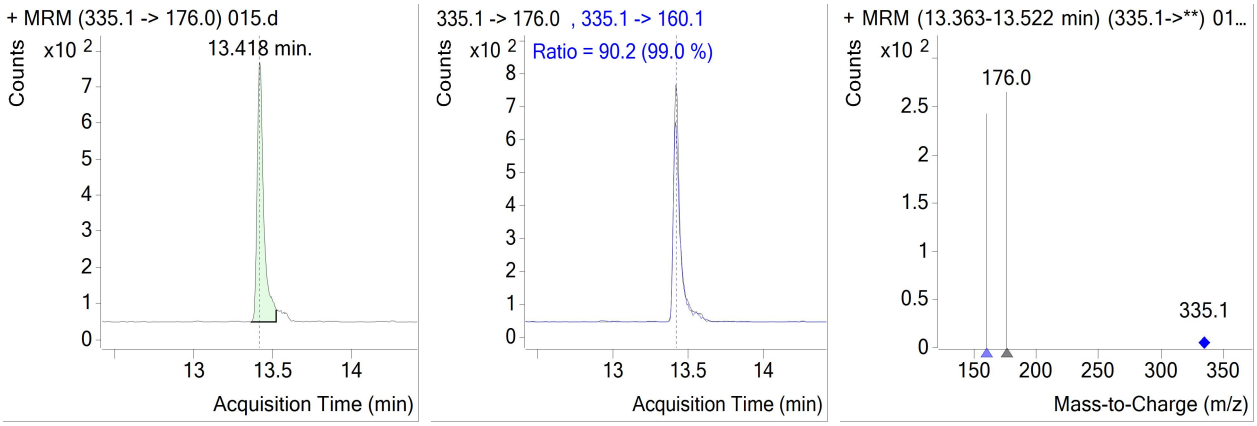
D. Enrofloxacin (ENR)







E. Doxycycline (DXY)



F. Penicillin G (PnG)

Method performance evaluation results

The analytical method validation in beef and poultry meat matrices was evaluated in-house as per the criteria stated in EU Commission Decision 2002/657/EC (C.D., 2002) and Codex Alimentarius commission (CAC) guidelines (Codex, 2014). Compound identification in the sample matrices was done by the presence of two ion transitions at the same RT comparing with those of the corresponding antimicrobial standards and ± 0.1 min absolute deviation (C.D., 2002; SANTE, 2019). TIC and sepaate individual chromatogram are shown in Fig. 1, 2 and 3).

Specificity

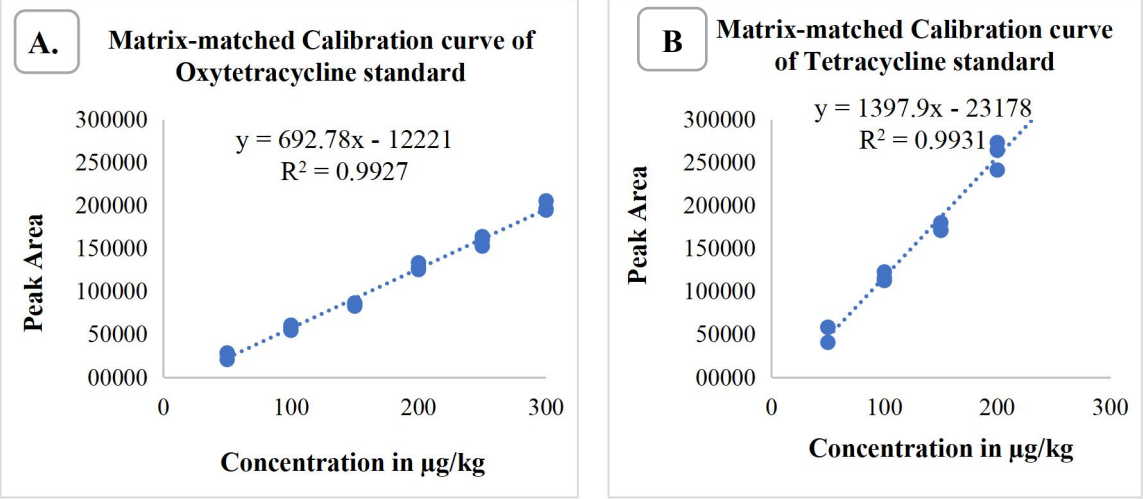
The chromatograms of the blank samples compared with that of spiked samples of target antimicrobials at the expected retention times for each analyte of interest (Peris-Vicente et al., 2015) to demonstrate selectivity of the analytical procedure. The result of the assay demonstrated that no significant interfering peaks at the retention time windows for all of the target antimicrobials that might produce a false-positive signal, demonstrating adequate selectivity.

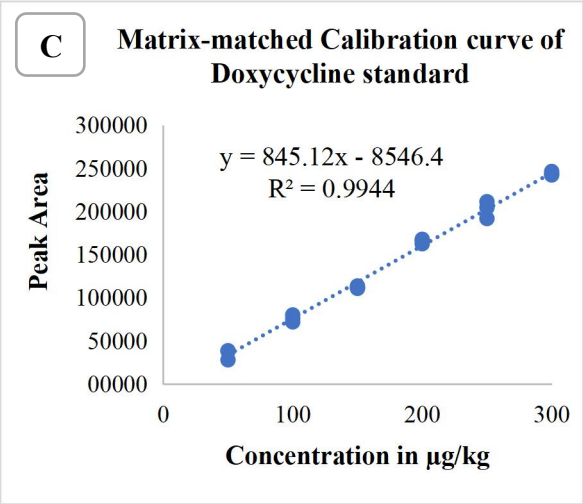
Linearity

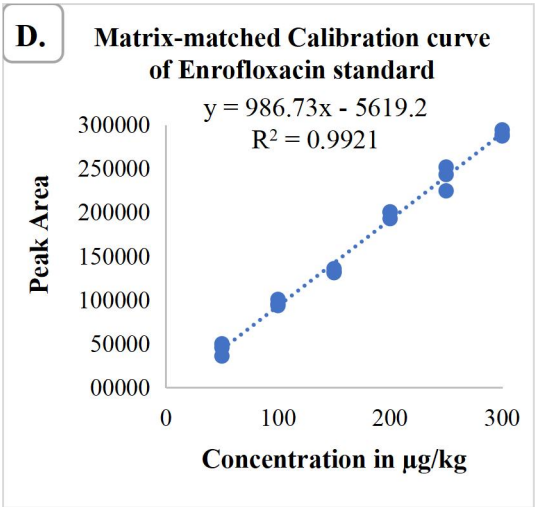
The calibration curves showed very good correlation of linearity over the range of the concentrations used in constructing the curves, characterized by a high coefficient of determination ($r^2 \geq 0.99$). The regression equation ($y = ax \pm b$) and the determination coefficient (r^2) were assessed by the least squares method. The y is the response signal or peak, and x the concentration of standard solution in µg/kg. The method quantifies antimicrobial residues in a linear range starting from 50 to 300 µ/kg. Figure 3 (A to D) presented sketch of representative matrix-matched calibration curve of each antimicrobial residues. Certainly, we can assume that this analytical method is linear for all targeted analytes in the selected concentration ranges (Jammoul and El Darra, 2019).

Figure 4.

Matrix-matched calibration curves of the standards for targeted antimicrobials at 50, 100, 150, 200, 250 and 300 ppb: Oxytetracycline (A), Tetracycline (B), Doxycycline (C), Enrofloxacine (D) (Partial view)







Certainly, we can assume that this analytical method is linear for all targeted analytes in the selected concentration ranges (Jammoul and El Darra, 2019) (Additional calibration curves were included in Annexe G).

Accuracy/recovery/ and precision

Regarding the recovery rate, it was determined by spiking of beef and poultry matrices at three levels 50, 100 and 150 µg/kg for each of the antimicrobials with the exception of penicillin G, which was at 25, 50 and 75 µg/kg levels with respect to MRLs of each antimicrobials. Referring to results of recoveries of the in-house-validated method, out of the six antimicrobials tested for their recoveries spiked in triplicate at three levels, the mean recovery rates obtained over the three days at three spike levels were very satisfactory, ranging from 93.9 % to 108.4% (Table 8). Therefore, the obtained values were acceptable for the method within the range (80 – 120%) as recommended by (C.D., 2002, E.C, 2021).

Table 8.

Accuracy and Precision of In-house-Validated Method (For Beef Matrices)

Analytes	Mean % Recoveries of 3	Mean Precision

	spiked concentrations (µg/kg)			Repeatability (% RSD_r) (µg/kg)			Within lab reproducibility (% RSD_R) (µg/kg)		
	50	100	150	50	100	150	50	100	150
SDZ	97.9	101.2	99.0	3.7	2.8	3.9	9.6	8.7	5.3
OTC	102.6	100.3	98.0	5.2	2.9	7.1	12.4	9.5	4.6
TTC	108.4	106.6	97.2	7.1	2.1	2.2	9.8	7.5	5.1
DXY	93.9	95.9	100.6	6.3	1.8	5.1	17.2	10.0	6.2
ENR	102.1	105.7	100.1	7.8	4.3	3.5	10.3	4.7	4.4
PnG	95.5	95.9	95.8	11.0	3.9	4.9	16.5	12.8	7.5

The repeatability (RSD_r) or intra-assay precision and within laboratory reproducibility (RSD_R) or inter-assay precision of the executed method showed ranges from 1.83 % to 11.00 % and from 4.44 to 17.2 % respectively. All the registered values of repeatability and reproducibility are under 23% and in harmony with the regulation 2002/657/EC or EU 2021/808 and less than 20% as per CAC/GL 71-2009. Hence, it can be confident that, accuracy and precision results obtained in the procedure were within the limits laid down by Codex and EU guidelines (C.D, 2002; CAC, 2009 and EU, 2021) and suitable to detect and quantify residues of the target antimicrobials proposed to be studied.

Relative matrix effect

The matrix effect (ME) or matrix factor (MF), which is produced by different matrix components co-exist with the analytes, would cause signal suppression or enhancement during ESI step. In this experiment, the matrix effect obtained for the six antimicrobials ranges from -19.36 % to 26.03 % for SDZ and DXY having the highest suppression and the highest signal enhancement respectively (Table 6). As per the EC 2021/808 EU guideline, the matrix effect should not greater than 20%. Therefore, the value of MF for DXY in beef sample was enhanced and out of the limit.

Limits of detection and Limit of quantification

Limit of detection (LOD) or method detection limit (MDL) and LOQ are not requirements for the determination of residues of authorized substances because legally

allowed veterinary drugs are monitored around the established legal limits (MRLs). Here, LOD and LOQ were determined as per the Eurachem guideline (Magnusson and Örnemark, 2014). The LOD results ranging from 1.59 to 2.53 µg/kg and LOQ ranging from 5.95 to 8.44 µg/kg were obtained in this particular study (Table 12).

Decision limits and detection capabilities

The decision limits (CCα) and detection capabilities (CCβ) were estimated by calibration curve procedure according to ISO 11843-2. The results obtained are within the limits laid down by Codex and EU guidelines (CAC/GL 71-2014 and EU 2021/808). The CCα and CCβ values obtained proved to be reliable for confirmatory analysis of the target antimicrobials at or around EU MRLs or at levels, much below the codex recommended MRLVDs (Table 9)

Table 9.

LOD, LOQ, CCα, CCβ and maximum residue limit for veterinary drug (MRLVDs) in raw beef and eviscerated poultry meat samples

Analytes	**LOD (µg/kg)**		**LOQ (µg/kg)**		**CCα (µg/kg)**		**CCβ (µg/kg)**		**MRL (µg/kg)**
	Beef	Poultry	Beef	Poultry	Beef	Poultry	Beef	Poultry	
SDZ	1.79	2.79	5.95	9.31	110.67	118.6	119.12	134.29	100
SDM	--	1.16	--	5.52	--	114.69	--	124.03	
OTC	2.33	2.72	7.76	9.06	108.93	117.97	121.58	132.03	100
TTC	1.99	1.99	6.62	6.64	106.4	111	116.4	121.8	100
DXY	2.39	3.11	7.95	10.36	107.57	118.39	117.24	133.98	100
ENR	1.99	3.20	6.63	10.68	107.78	112.82	115.9	119.84	100
PnG	2.53	--	8.44	--	56.94	--	61.42	--	50

Method Applicability to Real Samples (detection and quantification of residues)

Compound identification

The validated method then applied for the analysis of 180 beef and 120 chicken muscle samples collected from selected butcher houses and supermarkets of Addis Ababa city and 'Bishoftu' town and the samples were analyzed for six different antimicrobials (Table 10). For the screening of the targeted antimicrobial residues in unknown samples, for suspected potential peaks in the chromatogram reading, peak extraction were done and checked for the presence of two transitions at the same retention time for each targeted compounds. For confirmatory identification, two mass transitions must be present, for a peak at the same RT, which correspond to that of calibration solutions of the standards (relative deviations of ± 2.5 % and an absolute deviation of 0.1 min in RT were acceptable) (C.D., 2002 and EU, 2021). The ion ratio (qualifier /quantifier) or relative intensities of the most abundant ion must be within the acceptance criteria for ion ratio, typically not more than ± 25% (C.D, 2002; CAC, 2014 and EU, 2021). Accordingly, positive identification was confirmed only for those mass/ion transitions that simultaneously meet both the retention time and relative ion ratio criteria.

Occurrence of antimicrobial residues in raw beef and poultry muscle samples

The prevalence result of beef muscle samples showed that 26/180 (14.44 %) of the samples were positive at least for one of the six antimicrobial residues assessed (Table 10). Out of the positive samples, 11.67% contained one type of antimicrobial residues and 2.78% of the samples had multi residues, whereas none of the six antimicrobial residues were detected in 85.56 % of the samples. From 120 eviscerated poultry meat samples, 64/120 (53.33 %) were contaminated with at least with one of the six antimicrobial residues and 19/120 (15.83 %) were positive for multiclass residues and no detectable residues in 73/120 (60.83%) of the samples.

Table 10.

Occurrence of Antimicrobial Residues

Sample type	Antimicrobial residues	Samples with*

(number tested)	Single residue	More than one residues	ND residues
Beef muscle (n = 180)	21 (11.67%)	5 (2.78%)	154 (85.56%)
Chicken (n = 120)	47 (39.17%)	19 (15.83%)	73 (60.83%)

*ND, none detectible residues, samples found to contain zero or below the method LOQ

In this recent study, it was depicted that from the six antimicrobial residues assessed in raw beef muscle samples, OTC, TTC and SDZ account for 10.55 %, 2.78 % and 1.11% of the prevalence respectively from higher to lower level of occurrence. However, there were no detectable level of residues of ENR, DXY and PnG in the two study areas (Table 11). 14.54% and 14.29% of the tested beef muscle and 10.91% and 48.33% of poultry meat samples were positive for antimicrobial residues in Addis Ababa and Bishoftu town respectively. Specifically, 10.91% and 10% of the tested samples were positive for OTC in Addis Ababa and Bishoftu respectively. Concerning TTC residues, all of 2.78% of the residues were occur as a dual contamination with OTC.

Table 11.

Prevalence of Antimicrobial Residues in Beef Meat in the Two Study Areas

Sample collection area (numbers tested)	Antimicrobial residues prevalence (Test +ve)						Total prevalence (%)
	SDZ (%)	OTC (%)	TTC (%)	ENR (%)	DXY (%)	PnG (%)	
Addis Ababa (110)	2 (1.82)	12 (10.91)	2 (1.82)	0 (0.00)	0 (0.00)	0 (0.00)	16 (14. 54)
Bishoftu (70)	0 (0.0)	7 (10.0)	3 (4.29)	0 (0.00)	0 (0.00)	0 (0.00)	10 (14.29)
Total (n= 180)	**2 (1.11)**	**19 (10.55)**	**5 (2.78)**	**0 (0.00)**	**0 (0.00)**	**0 (0.00)**	**26 (14.44)**

Table 12.

Prevalence of Antimicrobial Residues in Poultry Meat in the Two Study Areas

Sample collection area (numbers tested)	Antimicrobial residues prevalence (Test +ve)						Total prevalence (%)
	SDZ (%)	SDM (%)	OTC (%)	TTC (%)	ENR (%)	DXY (%)	
Addis Ababa (55)	0 (0.00)	0 (0.00)	4 (7.27)	0 (0.0%)	0 (0.00)	2 (3.64)	6 (10.91)
Bishoftu (65)	4 (6.15)	0 (0.00)	20 (30.77)	0 (0.0%)	22 (33.85)	12 (18.46)	58 (89.23)
Total (n= 120)	**4 (3.33)**	**0 (0.0)**	**24 (20.0)**	**0 (0.0)**	**22 (18.33)**	**14 (11.67)**	**64 (53.33)**

Quantification of residues level in the study samples

Concerning the levels of antimicrobials found in beef muscle tissue, SDZ residue ranges from 5.2 to 11.5 µg/kg, OTC from 9.1 to 41.2 and TTC from 14.7 to 17.5 µg/kg were recorded in samples collected from Addis Ababa. In the capital city a mean concentration of 8.4, 22.0 and 16.1 µg/kg of SDZ, OTC and TTC residues were determined. Where as in Bishoftu town only OTC and TTC residues were quantified with mean concentration of 27.6 µg/kg and 13.7 µg/kg respectively (Table 12). In the present study, all residue concentrations of SDZ, OTC and TTC determined were much lower than the MRLs established by either EU (100 µg/kg) (EC, 2010) or Codex (200 µg/kg) (CAC, 2015 and CAC, 2018) guidelines for SDZ, OTC and TTC residues reported to be found the muscle samples. Thus, all the tested samples detected to be positive and all samples with none-detectable levels of antimicrobial residues were safe and acceptable for human consumption.

Table 13.

Levels of antimicrobial residues determined in beef tissue the two study areas

Concentration of residues (µg/kg)	Addis Ababa (n=110)			Bishoftu (n=70)	
	Sulfonamides	Tetracyclines		Tetracyclines	
	SDZ	OTC	TTC	OTC	TTC
Minimum	5.2	9.1	14.7	8.2	10.8
Maximum	11.5	41.2	17.5	46.3	18.0
Mean	8.4	22.0	16.1	27.6	13.7

Range	5.2-11.5	9.1-41.2	14.7-17.5	8.2-46.3	10.8-18.0
Total no. (%)	**2 (1.82)**	**13 (11.82)**	**2 (1.82)**	**7 (10)**	**3 (4.29)**

Table 14.

Levels of Antimicrobial Residues Quantified in Poultry Tissue Samples

Concentration of residues (µg/kg)	**Antimicrobial Types and Residue Levels**			
	Sulfonamides	**Tetracyclines**		**Quinolones**
	SDZ	**OTC**	**DXY**	**ENR**
Minimum	9.25	9.60	10.76	15.12
Maximum	13.29	145.69	22.56	99.08
Mean	11.09	26.28	28.79	407.13
Range	9.25-13.29	9.60-145.69	10.76-28.79	15.12-407.13
Total no. (%)	**4 (3.33%)**	**24 (20.0%)**	**14 (11.67)**	**22 (18.3)**

The levels of antimicrobials found in eviscerated poultry meat tissue depicted that, SDZ ranges from 9.25 to 13.29 µg/kg, OTC from 9.60 to 145.69, DXY from 10.76 to 28.5 µg/kg and ENR from 15.12 to 407.13. In the poultry meat samples, all residue concentrations of SDZ, and DXY quantified were lower than the MRLs established by either EU (100 µg/kg) (EC, 2010) or Codex (200 µg/kg) (CAC, 2015 and CAC, 2018) guidelines. Whereas, from poultry meat samples 1 (0.8%) and 8 (6.67%) were quantified to contain antimicrobial residues of OTC and ENR above EU MRLs respectively. Thus, a total of 9 (7.5%) poultry meat samples were unsafe and unacceptable for human consumption (Table 14) and Figure 6.

Figure 5.

Occurrence of Antimicrobial Residues in Eviscerated Poultry Meat Muscle

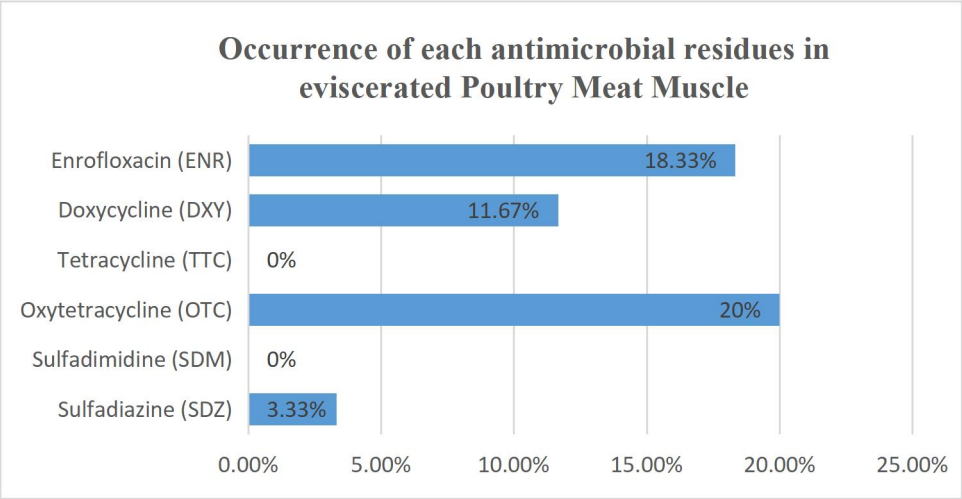
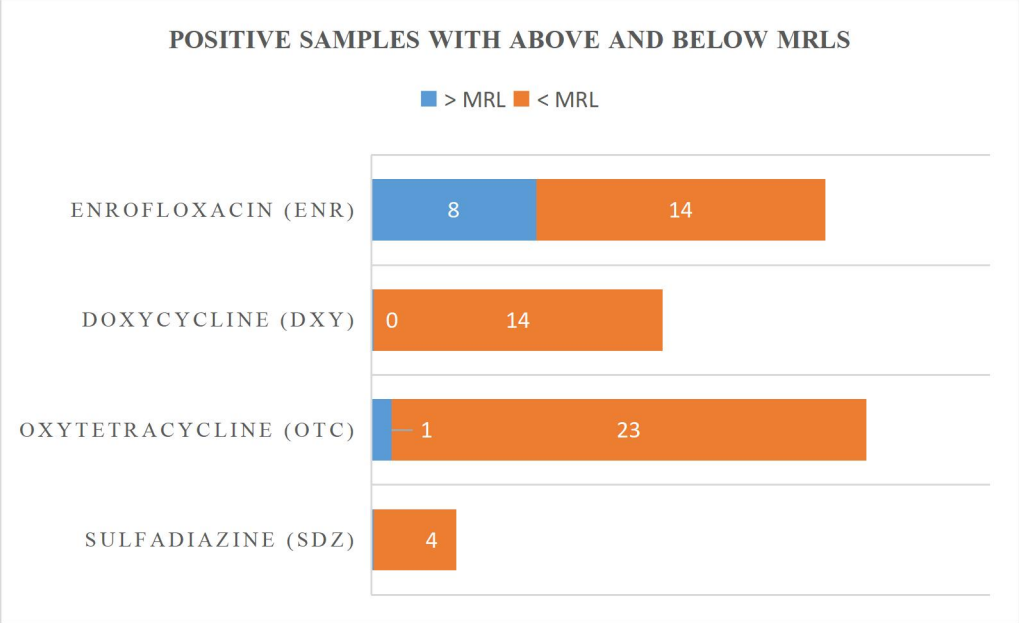


Figure 6.

Positive Samples With Above and Below MRLs



CHAPTER V

Discussion

Detection and determination of multidrug residues from beef muscle was reported for the first time in Ethiopia. In this particular research, from four classes of veterinary antimicrobials, six antimicrobial drug residues were preliminarily assayed using SPE as a sample preparation technique and analyzed by UHPLC-MS/MS. The OTC residue occurrence, presented in this study was far lower than in the previous study

93.8 %, 37.5 % and 82.1 % in Addis Ababa, Bishoftu and Adama respectively, which was reported by Bedada and Zewde (2012).

The current study, which was carried out on beef samples collected from Butcher shops and supermarkets found in the central part of the country, presented OTC residue of 22.0 µg/kg and 27.6 µg/kg in Addis Ababa and Bishoftu respectively. The study conducted by Bedada and Zewde (2012) on beef samples collected from slaughterhouses depicted 108.34 and 15.92 µg/kg OTC residues, in the aforementioned cities respectively. Since the research done on a single antimicrobial residue, there were no reports on SDZ and TTC residues and this makes our research to be the first of its kind in the study areas.

With respect to sample compliance, Bedada and Zewde (2012) have reported that all the beef samples collected from Bishoftu town were found compliant, similar to the findings of our current research. In the contrary, the same authors recorded oxytetracycline residues above EU-established MRL (100µg/kg) at about 48 % in beef muscle collected from Addis Ababa. However, no information was available regarding the performance of the test methods utilized in the laboratory setup where the test was performed. In another research, which was done by Agmas and Adugna (2018) in and around Debre Tabor and Bahir Dar towns), out of 250 beef cattle slaughtered 76.4 % tested positive for antimicrobial residues. The research was done using Premi® test kits and significantly higher antimicrobial residue prevalence was reported than the present findings. Hence, it must be noted that such a screening method is inherently limited in providing the definitive identification of the specific antimicrobial or class of antimicrobials, which contributed to the presumptive positive results.

A similar study conducted in Lebanon on beef meat samples using LC-MS/MS for targeted antimicrobials residue assaying showed residue occurrence of 16 %, which is comparable with the current study. Besides, 84 % of beef samples contain no detectable or zero residues which were almost similar to our recent research findings

(85%) and all of the tested beef samples destined for retail outlets were found to be compliant and fit for human consumption (Mokh et al., 2020).

A study conducted in Cameroon on beef samples destined for public consumption, using liquid chromatography with a triple quad mass spectrometer (LC-MS/MS), indicated that 20.3% (41/202) of the samples contained residues of interest compounds. The mean determined residue concentrations (OTC = 240 μg/kg and PnG = 17.58 μg/ kg) were significantly higher than the present findings (Ngom et al., 2017). Conversely, a study conducted only on OTC residue in 60 beef samples in Tanzania using the LC-MS method had recorded much lower mean residue levels (0.69 ± 0.09 μg/kg; 35%) compared with our current research (Mgonja et al., 2017). A similar study reported in southwestern Nigeria also revealed a low level of oxytetracycline and penicillin-G residues (Adesokan et al., 2013).

According to the U.S. national residues program for meat, out of 9,057 beef cow carcasses that were analyzed in 2019 by FSIS labs, only 24 (0.26 %) of the samples residues violation found. The data obtained from the confirmatory method of analysis such as LC-MS/MS conferred very low levels of violative residues. (USDA, 2019). Similarly, in the European food safety authority's annual residue-monitoring program for 2019 in the EU member states, only a few non-compliant residues occurred. Out of the 22,109 numbers of bovine samples analyzed for one or more substances, only 56 (0.25%) non-compliant samples were reported (EFSA, 2021).

CHAPTER VI

Conclusion and Recommendations

Conclusions

A multiclass ultra-high-performance LC with mass spectrometer method of analysis was validated successfully for the simultaneous screening and quantification/ determination of antimicrobial residues in beef and poultry meat muscle. The method validation parameters demonstrate very good linearity, high recovery, excellent repeatability, outstanding reproducibility and specificity of the analytical method. Moreover, the method fulfills method validation criteria of European commission 2021/808 and Codex Alimentarius Commission guidelines and can be used for both screening and confirmatory analysis of veterinary antimicrobial residues. Hence, applicability of the method for monitoring of SDZ, SDM, OTC, TTC, ENR, DXY and PnG antimicrobial residues in raw beef and eviscerated poultry meat samples was established.

The study revealed the occurrence and contamination level of raw beef samples with three types residues and poultry meat with four types of residues from the tested four families of veterinary antimicrobials, frequently used in animal health practice. Out of 180 beef samples analysed, 14.44% contained at least one of the six antimicrobial residues and 85.56 % were with none-detected residues. From the six antimicrobials, three types of residues were detected and their levels determined in beef matrices from 5.2 to 46.3 μ g/kg. However all beef samples with confirmed detectable levels of antimicrobial residues were below the MRLs and compliant with the EU maximum residue limits.

However, the assayed eviscerated poultry meat samples demonstrated that, more than half of the tested poultry meat samples (53.33%) were positive for antimicrobial residues and 46.67% reported to be with none detectable levels of residues. In contrary to the beef samples, 7.5 % of poultry meat samples found to contain antimicrobial residues above the MRLs of EU standards. From these findings, it can be concluded that,

at the poultry farms in the study area utilization of uncontrolled level of commonly used veterinary antimicrobials.

Even if low level of residues occurrence was recorded in the findings of tested beef samples, this may not necessarily guarantee the presence of legitimate and judicial use of veterinary antibiotics in food animals. On the other hand, more occurrence of residues positive cases and high levels of antimicrobial concentrations were detected in eviscerated poultry meat samples.

Recommendations

In line with the above conclusion, the following recommendations are forwarded:

- The methods validated in this study are practical, efficient and can be effectively used by the labs for simultaneous screening and confirmatory analysis of antimicrobial residues in food of animal origin like beef and poultry muscle meat
- Multiclass antimicrobial residues testing methods like UHPLC-MS/MS provide trustworthy results in regulatory decision-making in view of that, it should be used for residue monitoring activities in developing countries like Ethiopia
- To keep the level of antimicrobial residues below MRLs, Planned residue monitoring and controlling programs should be implemented in the study areas
- The use of veterinary antimicrobials at the poultry production farms should respect withdrawal period of the drugs to decrease the concentration of residues in poultry meat below the tolerance level (MRLs)
- To reduce the level of antimicrobial residues in poultry meat, there should be an intervention via adult education and awareness creation programs on rational use of veterinary antimicrobials
- The study stressed on performing planned residue monitoring activities in poultry farms and regular sampling and residues assaying of poultry meat samples
- Introduction of cost-effective and sensitive screening methods of residue analysis are required to augment LC-MS/MS method and detect the potential occurrence of unsafe levels of antimicrobial residues

- This research is limited in terms of study area coverage; further compressive research should be carried out to analyze large number of representative samples gathered from different parts of Ethiopia.

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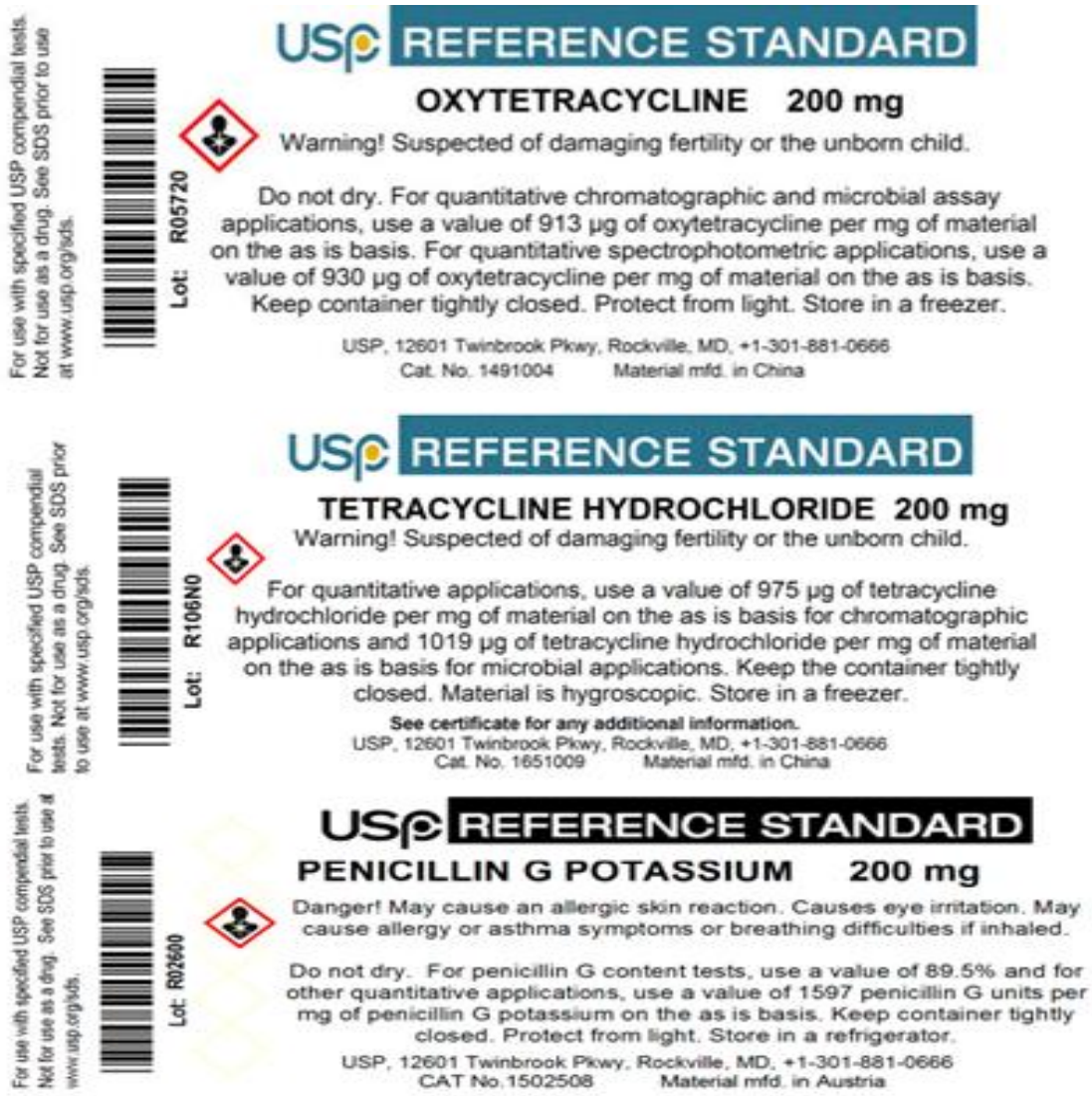
APPENDICES

Appendix A

Detail information of USP Reference standards used for Oxytetracycline



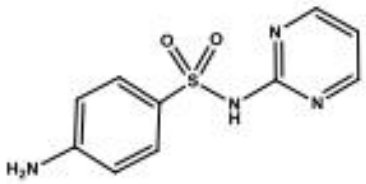
Appendix B

Detail information of USP Reference standards used for sulfadiazine

U.S. Pharmacopeia
The Standard of Quality™

USP Certificate

Sulfadiazine
LOT K0K193



Molecular Formula
$C_{10}H_{10}N_4O_2S$

Molecular Weight
250.28

CAS Number
68-35-9

LABEL TEXT

Teri L. Toth
Quality Assurance

Annex C

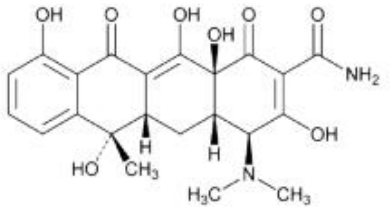
Detail information of USP Reference standards used for sulfadiazine

Certificate

TETRACYCLINE HYDROCHLORIDE

((4S,4aS,5aS,6S,12aS)-4-(Dimethylamino)- 1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-2-naphthacenecarboxamide monohydrochloride)

USP Catalog No.:	1651009
USP Lot No.:	R106N0



CAS No.:	64-75-5
Molecular Formula:	$C_{22}H_{24}N_2O_8 \cdot HCl$
Molecular Weight:	480.90

LABEL TEXT

For use with specified USP compendial tests and not for use as a drug. See SDS prior to use at www.usp.org/sds.

Lot: R106N0

USP REFERENCE STANDARD

TETRACYCLINE HYDROCHLORIDE 200 mg

Warning! Suspected of damaging fertility or the unborn child.

For quantitative applications, use a value of 975 µg of tetracycline hydrochloride per mg of material on the as is basis for chromatographic applications and 1019 µg of tetracycline hydrochloride per mg of material on the as is basis for microbial applications. Keep the container tightly closed. Material is hygroscopic. Store in a freezer.

See certificate for any additional information.

USP, 12601 Twinbrook Pkwy, Rockville, MD, +1-301-881-0666
Cat. No. 1651009 Material mfd. in China

Obtain special instructions before use. Do not handle until all safety precautions have been read and understood. Wear protective gloves/protective clothing/eye protection/face protection. If exposed or concerned: Get medical advice/attention. Store locked up. Dispose of contents/container in accordance with local/regional/national/international regulations.

Teri L. Toth

Quality Assurance

Annex D

Mobile Phase and Extraction solutions preparations

Preparations of solutions

Aqueous mobile phase: (Water, 0.1% Formic Acid): 1.0 mL of formic acid was added into almost half filled 1.0 L ultrapure deionized water in a 1-liter volumetric flask. The mobile phase degassed in an ultrasonic water bath for 10 minutes then transferred to reservoir A of the UHPLC.

Organic mobile phase (Acetonitrile, 0.1% Formic Acid): Again 1.0 mL of formic acid was pipetted into a half filled 1.0 L volumetric flask and brought to the volume using acetonitrile. This was degassed and transferred to the organic reservoir of the UHPLC.

a) **Extraction Solution (Acidified acetonitrile + McIlvaine Buffer/0.1 M Na_2EDTA):**
   - **0.1% Formic Acid in acetonitrile**: About 0.40 ml of formic acid was pipetted and mixed with 400 mL of acetonitrile in graduated cylinder and then transferred to a dispenser bottle for storage.
   - **McIlvaine buffer** (mixed citrate-phosphate): 14.21 g anhydrous dibasic sodium phosphate and 9.605g anhydrous citric acid were separately dissolved well each in 500 ml de-ionized water. Then 308 ml citric acid solution (0.1M) and 192 mL phosphate solution (0.2M) were mixed carefully in Duran bottle (pH was maintained at 4.00 ± 0.05)
   - **McIlvaine Buffer/0.1 M Na_2EDTA**: 18.61 g disodium EDTA dihydrate added in 500mL McIlvaine buffer and sonicated.

b) **Diluent (**80:20 Water/Acetonitrile**)**: 80 mL of deionized water and 20 mL of acetonitrile were measured using graduated cylinders and combined in a 100 ml flask.

Annex E

Preparation of standard solution

- Stock standard solutions were prepared at concentrations corresponding to 1.0 mg/mL (1000µg/ml) taking in to account stability and solubility of the drug in the solvent. The standard solutions were prepared in methanol for each tetracycline, and in de-ionized water for penicillin G by transferring 10.0 mg equivalent of the base materials quantitatively in to a 10.0 ml class A volumetric flasks separately, and diluted to volume with suitable solvents and stored in amber vials at ≤ -20◦C.

- Intermediate standard solution for penicillin G (20 ng/µl) was prepared by transferring 200µl stock and diluting to 10 ml final volume with water. Composite intermediate standard solution for tetracyclines was prepared by pipetting 400µl aliquot of stock and diluting in a 10.0ml volumetric flask with methanol to 40ng/µl.

- A working standard (WS) was made by pipetting 1.0 ml of intermediate solutions into a 10 ml volumetric flask and diluting to the mark with diluent (80:20 water/Acetonitrile) giving final concentrations of 2 ng/µl and 4 ng/µl for pen G and for TCs respectively.

Annex F

Table 15. Performance requirements for precision and recovery

Concentration µg/kg	Mean % Recovery		Reproducibility (repeatability) Limits, CV (%)*		References
	Codex	EU	Codex	EU	
≤ 1	50-120	50-120	36 (35)	30 (20)**	CAC/GL 71-20014 and EU 2021/808
1 to 10	60-120	70-120	32 (30)	30 (20)**	
> 10 to 100	70-120	80-120	22 (20)	25 (16)	
> 100 to 1000	70-110	80-120	18 (15)	22 (15)	
≥1000	70-110	80-120	14 (10)	16 (10)	

*The coefficient of variation (CV %) shall not exceed the level calculated by the Horwitz Equation: ($\%CV_R = 2^{(1-0,5 \log C)}$) *Repeatability should be $\leq \frac{2}{3} CV_R$, and for **C < 100 µg/kg, the CV (%) presented is a guideline and should be as low as reasonably possible.

Table 16. Maximum permitted tolerances for relative ion intensities for LC-MS/MS

Relative ion intensity (% of base peak)	LC-MS/MS (% relative deviation)		References
	Codex	EU**	
> 50	≤ 20%	±20%	CAC/GL 71-2009 and EU 2002/657
>20% to 50%	≤ 25%	±25%	
>10% to 20%	≤ 30%	±30%	
≤10%	≤ 50%	±50%	

**According to revised EU guideline (EU2021/808), all acceptable ion ratios shall be ±40%

Annex G

Matrix-Matched Calıbratıon Curves of Penıcıllın G and Sulfadiazıne antibiotics

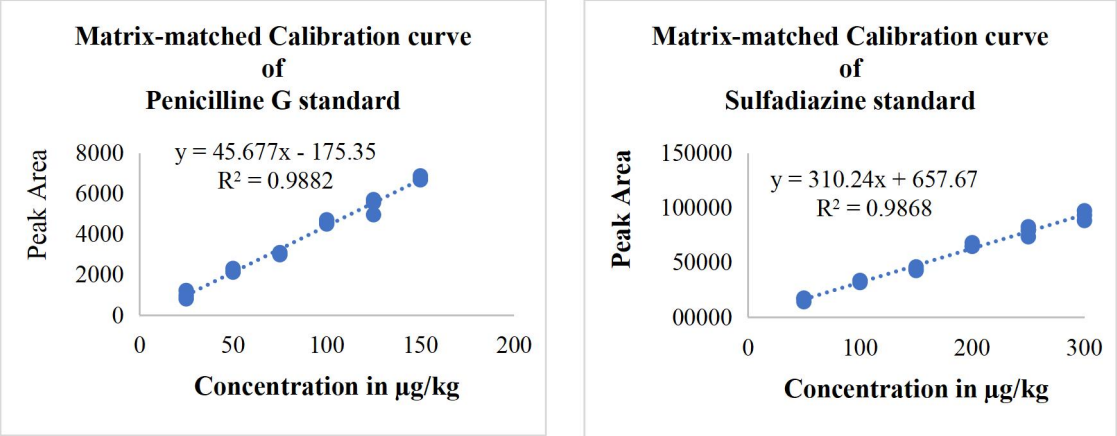


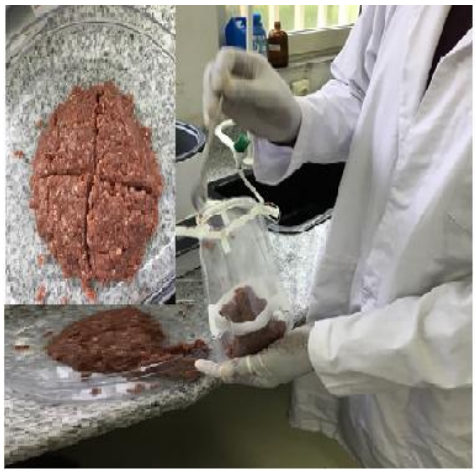
Figure 7 Matrix-matched calibration curves for targeted antimicrobials (Partial view)

Annex H

Sample extraction and cleanup procedures

1. Remove the fat and connective tissues and mince the sample rapidly with a blender

2. Divide the homogenized sample in to halves and preserve frozen (< -20 °C) until analysis

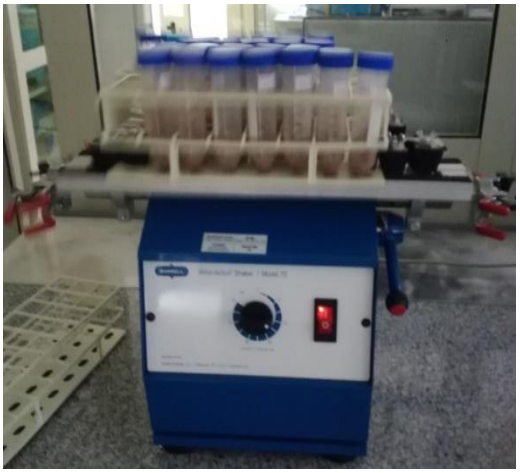


3. Accurately weigh 4.00 ± 0.005 g of the minced sample into a 50 ml centrifuge tubes

4. Spike control sample at this stage with appropriate volume of working standard solutions. Briefly vortex and allow spiked sample to stand for 30 mınutes in a dark

5. Add 2 ml of McIlvaine Buffer/0.1 M Na_2EDTA and 8 ml acetonitrile into the sample

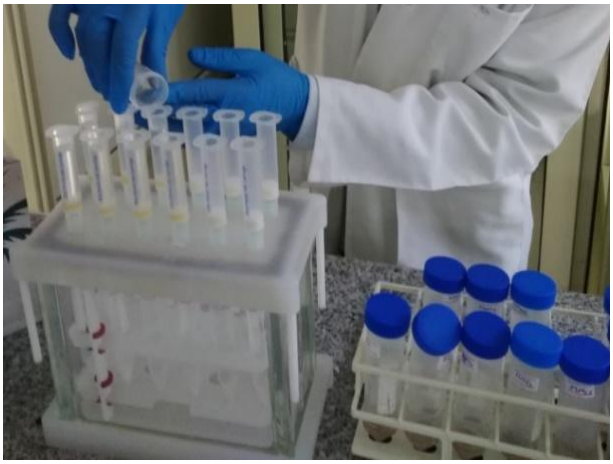
6. Shake mechanically for 15 minutes



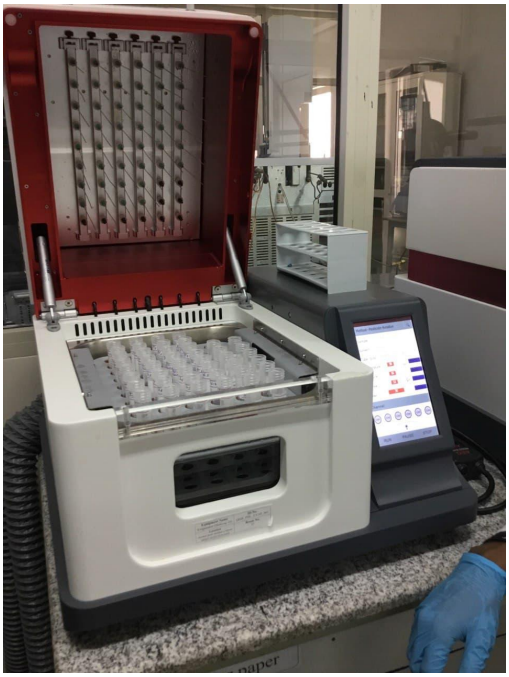
7. Centrifuge at 4º C in a cooling centrifuge for 15 min at 4,500 rpm

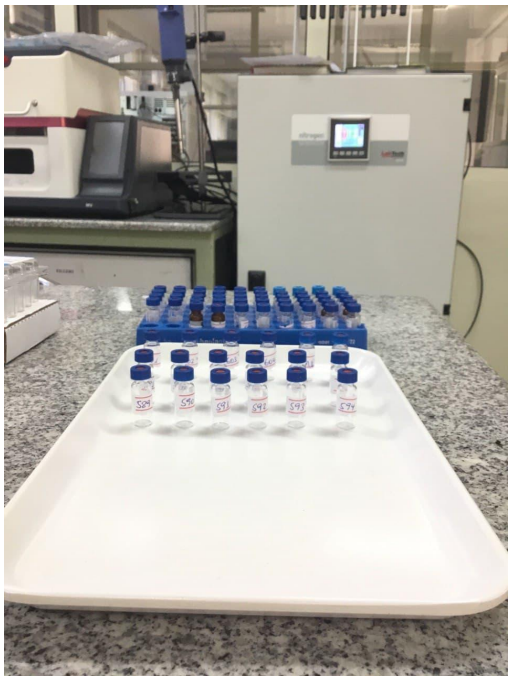
8. Mount Oasis PRiME HLB (6cc, 200 mg) cartridges on to the vacuum manifold

9. Carefully decant the supernatant into Oasis PRiME HLB cartridges allowing the sample to pass through gravity into an empty pre-labeled 15 mL centrifuge tube



10. Transfer about 5 mL of the clean extract (equivalent to 2 g sample) in to a conical 15 ml tubes and evaporate under stream of N_2 gas at 40-45ºC to ≤ 0.1ml

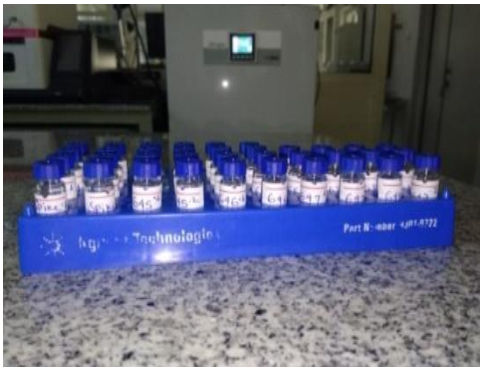




11. Reconstitute the residue with mixture of water/acetonitrile (8:2 v/v) to 1ml

12. Vortex mix, and centrifuge for 5 min.

13. Cautiously transfer about 0.5ml of the extract into labeled auto-sampler vials for injection



CURRICULUM VITAE

Appendices I

Name	Belachew Bacha	Surname	Hirpessa
Place of Birth	Addis Ababa	Date of Birth	June 20, 1980
Nationality	Ethiopia	E-Mail	bellnext@gmail.com
Address	Tele:- Mob. +251912387870, Landline: 251-0114-717270, P.O. Box 31303 Addis Ababa, Ethiopia.		

Educational background	**Names of Educational Institutions**	**Graduation Year**	**Awarded Degree**
Food Hygiene and Technology	Near East University, Nicosia, Turkish Republic of North Cyprus	2022	Ph.D.
Obstetrics and Gynecology	Addis Ababa University , Addis Ababa, Ethiopia	2007	M.Sc.
Veterinary Medicine	Addis Ababa University , Addis Ababa, Ethiopia	2005	DVM
High School	Ayer-tena senior secondary high School	2004	High school Certificate

Employment History	**Name of Organization**	**From**	**Up to**
Academic rank Assistant Professor, Teaching, Work as Academic Programs officer (APO) of Wollega University, as dean of Graduate Studies.	Wollega University	2007	2013
Director for Physicochemical laboratories of Quality Control Center of Ethiopian Agriculture Authority	Ethiopian Agriculture Authority, Former Veterinary Drug and Feed Administration and Control Authority	2014	Up to date

Training attended	Name of Awardıng organization	Year	Award
• Residues of veterinary medicines in food of animal origin, 16 hrs. modular courses of 3 months period	Veterınary Medicines Dırectorate of the Unıted Kıngdom (UK, VMD)	2021	Certificate
• Training on Residue and microbial testing in feed and food of animal origin	UNIDO in collaboratıon wıth SABI PLC.	2022 Sep. 05 – 09	Certificate
• Analysis of authorized Veterinary Antibiotic residues in products of animal origin. (Extensive Lab. Training courses).	RIKILT Wageningen University Research (WU-R) Institute of Food Safety, the Netherlands.	August 1 – 31/2020 (1 month)	Certificate
• Validation of methods for the analysis of authorized substances in products of animal origin. (Extensive Laboratory Training courses on)	RIKILT Wageningen University Research (WU-R) Institute of Food Safety, the Netherlands	February1 – April 30/ 2020 (two months)	Certificate
• Quality management system ISO/IEC 17027: 2017	Ethıopıan Standards Authorıty	2020	Certificate
• Training on 'Antibiotic residues analysis in food and feed'	RIKILT Wageningen University Research (WU-R)	November 17-25/2014	Certificate
• Training on management and leadership skills in organization	Ethiopian Management Institute	2016	Certificate
• Statistic as a tool for analytical laboratory, quality control and assurance, method validation,	JIJE LABOGLASS Pvt. Limited Company	2016	Certificate

traceability and uncertainty			
• Advanced Pharmaceutical Good Manufacturing Practice (GMP) Inspection	USAID/USP	2016	Certificate
• Basic Pharmaceutical Good Manufacturing Practice (GMP) Inspection (USAID/USP)	USAID/USP	2015	Certificate
• Laboratory Management System based on ISO/IEC 17025:2005	Ethiopian Standards Agency	2015	Certificate
• Workshop on "**Information retrieval system**" on 18/11/2013 at Bioinformatics center and ARIS cell,	Madras Veterinary College, Chennai- 600 007, India.	2013	Certificate
• CISCO International certificate of "IT Essentials: PC Hardware and Software"	Wollega University	Aug. – Sep. 31/ 2011	Certificate
• HDP- Nine Months training on (Teaching Methodologies, student assessments methods and classroom mgt. etc. …)	Wollega University	October 2009 – June 2010	Higher Diploma (HDP)
•			

Language	**Listening skill**	**Speaking skill**	**Writing skill**	**Reading skill**
Amharic	Native	Native	Native	Native
English	Excellent	Excellent	Excellent	Excellent
Turkish	Fairly	Fairly	Fairly	Fairly