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Antimicrobial Residue Analytical Methods and Its' Maximum Residue Limits in Chicken Muscle. A systematic Review

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SUMMARY: Poultry farmers use disease preventive and growth promoter antimicrobial for faster growth, to increase the rate of feed assimilation and to lower the incidence of mortality. Due to this Chicken muscle contain antimicrobial residues. Presence of residues in chicken muscle above MRL is recognized as an important food safety and health hazards. Drug residues are now monitored worldwide by many government and private contract laboratories to enforce regulations domestically and in international food trade. Drug analysis is challenging to develop a method for drugs residue analysis from chicken muscle due to the complexity of sample matrices and analytes various chemical properties. Hence reliable sample extraction methods are necessary to ensure food safety. There are various extraction and clean up methods for analysis of antimicrobial residue such as, liquid-liquid extraction and solid phase extraction. Promising extraction and pre-concentration techniques have been explored recently. Possible antimicrobial residue levels in chicken muscle must be determined through highly selective and sensitive methods. Today various advanced analytic methods capable of qualitative and quantitative measurement, such as ELISA, Charm II, TLC, GC, HPLC, LC-MS, LC-MS/MS and UHPLC/MS/MS are used to detect antimicrobial residue in chicken muscle. Antimicrobial residues prevalence in chicken muscle samples have different values in different countries. In Ethiopia; there is no enough information about prevalence and analysis of antimicrobial residues in chicken muscle. In Ethiopia antimicrobial residues prevalence in chicken muscle done by some researchers are at non-detectable level. To know well the situation of antimicrobial residue in chicken muscle; further investigation is required.

Key Words: **Analytical methods, Antimicrobial residues, Chicken muscle, Maximum drug residue limit, Sample extraction**

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1. INTRODUCTION

Over the past few decades poultry has gone through tremendous growth (Mund *et al*., 2017). Broiler chickens are considered among the largest sources of meat across the world. Demand for chicken meat has increased in every region of the world, particularly in developing and Asian countries. An increase in demand has put the farmer under continuous pressure to produce poultry (chicken) in the shortest period of time with maximum output (Muaz *et al*., 2018). It's considered as an important source of protein for human. It is easily digested and have palatable taste and is economically cheaper than red meat. It is of low calories, its fat contains essential fatty acids and its protein is good source of essential amino acids (Jayalakshmi *et al*., 2017)

The production of high quality poultry meat for consumption is very essential (Offiah & Adesiyun, 2015). A lot of food producing animals and birds receive antimicrobials for part or most their lives (Jayalakshmi *et al*., 2017). In poultry production, antimicrobials are essential to prevent and control infectious diseases. The use of antimicrobials for therapeutic, preventative and/or growth promotion purposes in chicken (broiler) is causing serious problems in human associated with the presence of residues in chicken muscle. The presence of residues in chicken muscle above the maximum levels or residue limits is recognized worldwide as an important food safety and health hazards(Kozarova *et al*., 2020). The misuse and incorrect application of antimicrobials carries the risk of their residues presence in muscle tissues of chicken, which can cause toxics and allergies in hypersensitive consumers (Marazuela & Bogialli, 2009).

Additionally, human exposure to high levels of antimicrobials residues from chicken muscle sources may aggravate immunological response in low immune individuals and influence negatively the intestinal gut microbiota (Jammoul & Darra, 2019) and become cause of economic losses in the food industry

especially by interfering with starter culture (Kozarova *et al*., 2020). The misuse of antimicrobials may trigger the development of resistant strains of bacteria, thus reducing the efficiency of antimicrobials used for chicken, leading to the treatment failure in chicken, and affecting negatively the chicken welfare. The spread of antimicrobial resistance is a problem of major concern globally. Therefore, the antimicrobials residues are being considered as public health hazards, since there is a concern about the transfer of antimicrobial resistant genes from chicken flora to human pathogens. Another risk comes from the transfer of antimicrobial resistant bacterial strains through the food chain (Jammoul & Darra, 2019).

The MRL is defined as the maximum concentration of a residue, resulting from the registered use of an agricultural or veterinary chemical that is recommended to be legally permitted or recognized as acceptable in or on a food, agricultural commodity, or animal feed. The concentration is expressed in milligrams per kilogram of the commodity (or milligrams per liter in the case of a liquid commodity (Kebede *et al*., 2014).

Antimicrobial residues are now monitored worldwide by many government and private contract laboratories to enforce regulations domestically and in international food trade. A common goal in drug analysis is to get acceptable results for many analytes by a cost-effective method in a single run. However, it is challenging to develop such a method for antimicrobial in chicken muscle tissues due to the complexity of sample matrices and diversity of analytes from various classes of chemical properties.Hence reliable sample extraction methods for rapid, selective and sensitive detection of these residues are necessary to ensure food safety (Jingcun *et al*., 2018). There are various extraction methods that have been used in antimicrobial residues analysis in chicken muscle samples, such as liquid-liquid extraction (LLE) and solid phase extraction (SPE) (Moyo & Tawanda, 2019).

In the past, antimicrobial analysis methods were developed for specific analytes or groups of closely related analytes with less selective instruments and extensive sample clean up steps, many single class methods had to be used for monitoring all the targeted drugs(Jingcun *et al*., 2018).

Possible antimicrobial residue levels in chicken muscle must be determined through highly selective and sensitive chemical methods. Various advanced analytic methods capable of quantitative measurement, such as Enzyme-Linked Immunosorbent Assay (ELISA), Charm II, Gas Chromatography (GC) and high performance liquid chromatography (HPLC), Liquid Chromatography Mass Spectrometry (LC-MS) and Liquid Chromatography Mass Spectrometry/ Mass Spectrometry (LC-MS/MS) are used to detect antimicrobial residues in chicken muscle. HPLC is preferred not only because of its capability to analyze thermolabile compounds, but also because it is a specific, certain and sensitive method (Nkechi *et al*., 2018).Recently, with the advance of ultra-high-performance separation and high sensitive and selective mass detection techniques, the single-class methods have been gradually replaced by Multiclass Multiresidue Methods (MMMs) like; Ultra-High Performance Liquid Chromatography/Mass Spectrometry/Mass Spectrometry (UHPLC/MS/MS) (Jingcun *et al*., 2018).

LC-MS/MS is widely used to quantify various antimicrobial residues in chicken muscle with good sensitivity and specificity. Tolerance limits and maximum residual limits have been established around the world and agencies to monitor the food supply to ensure that antimicrobial residue concentrations do not exceed the MRL levels. However, there is only little information about the residual analysis and levels of antimicrobial in chicken muscle in Ethiopia. The objective of this seminar is review analytical methods of analysis and its maximum residue limits (MRL) of antimicrobial residues inchicken muscle.

2. Analytical Methods of Antimicrobial Residues from Chicken Muscle

The most serious concern about excessive use of antimicrobials is the impact on the effectiveness of the human medicines as bacteria have started to become resistant to the most common antimicrobials. In order to combat this problem and protect the human consumers, the use of antimicrobials is tightly regulated. The European Union (EU) Council Directive 96/23/EC requires monitoring of certain antimicrobials in live animals and in animal products. To ensure that testing is carried out to the highest analytical standards unambiguous guidelines stipulate the rules for the analytical methods to be used for testing chicken muscle for the presence of residues and contaminants. Analytical methods employed for determination of antimicrobials must be capable of determining the residues below their MRLs. To fulfil the regulatory requirements it is necessary to employ sensitive, selective and reliable analytical methods (Bousova *et al*., 2013). The detection of antimicrobials residues in chicken muscle requires sample pre-processing, instrumentation method establishment and data analysis to evaluate the stability, precision, and sensitivity of the established method (Wang *et al*., 2021).

Antimicrobials are compounds characterized by a complex chemical structure that have very variable water solubility, low volatilization potential, several ionizable functional groups (amphoteric molecules) and different pKa values hence they have a low bioaccumulation potential. Antimicrobials may have different functionalities within the same molecule, making them either neutral, cationic, anionic, or zwitterionic under different pH conditions. Those properties of antimicrobials are affect the detection and quantification of analytes from a given sample. To overcome those challenges sample preparation (extraction and clean up techniques) are very important because most recent method of sample preparations are fast, easy and cheap for routine analysis of residues in chicken muscle. The latest trend in drug residue analysis

is the development of generic methods that are capable of monitoring a wide variety of compounds, belonging to different veterinary drug classes. Most extraction methods still making use of organic solvents may be completely eliminated in future. Currently greener solvents such ionic liquids are widely used in micro-extraction procedures as dispersive or extraction solvents according to their different solubility in DLLME. Electrochemical sensors and their relative detection strategies, with the advantages of high sensitivity, simplicity and rapid response, have attracted considerable attention in recent years (Babra & Nikita, 2019).

2.1.Sample Preparation (Extraction and Cleanup) Techniques

chicken muscle samples have a complex matrix and many endogenous interfering substances, making it impossible to directly detect antimicrobials residues (Wang *et al*., 2021). Hence reliable sample extraction methods for rapid, selective and sensitive detection of these residues are necessary to ensure food safety (Moyo & Tawanda, 2019). In the extraction technique mixture of substances are dissociated, by dissolving each component with one or other solvents which yields two phases; Raffinate Phase and Extract Phase (Patel *et al*., 2019).

There are various extraction methods that have been used in antimicrobials residues analysis in chicken muscle samples, such as liquid-liquid extraction (LLE) and solid phase extraction (SPE). These methods suffer a number of drawbacks even though they perform their tasks adequately. Both LLE and SPE are environmentally unfriendly due to the large amounts of organic solvents they use, they are time consuming and labor intensive. Another disadvantage of SPE is that cartridges are costly (Moyo & Tawanda, 2019).

Promising extraction and pre-concentration techniques for antimicrobial residues that have been explored recently by many researchers include; Dispersive Liquid-Liquid Microextraction (DLLME), Hollow Fiber Based Liquid-Phase Microextraction (HF-LPME) and Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) (Moyo & Tawanda, 2019), Matrix Solid Phase Dispersion (MSPD), Accelerated Solvent Extraction (ASE) (Patel *et al*., 2019); where the general trend is compliance with green chemistry principles. Antimicrobials residues occur at trace levels as low nanogram per gram hence the need to pre-concentrate (Moyo & Tawanda, 2019).

2.1.1. Liquid-Liquid Extraction

The LLE method has been used to extract antimicrobials residues from chicken muscle for nearly a decade (Wang et al., 2021). It is also known as solvent extraction refers to an operation in which the components of the liquid mixture are separated by contacting it with a suitable insoluble liquid solvent which preferentially dissolves one or more components. In this type of operation, the separation of the components of solution depends upon the unequal distribution of the components between two immiscible liquids (Patel *et al*., 2019).

The method is simple in operation but has disadvantages such as high reagent consumption, time consumption and chance of manual error. Moreover, toxic organic solvents are usually used in the LLE extraction process, as researchers must take protective measures to avoid physical harm. Different extraction reagents are used to extract antimicrobials residues from chicken muscle, including acetonitrile (ACN), Ethylene Diaminetetraacetic Acid Disodium Salt (EDTA)-succinate, 0.1% formic acid in aqueous solution of EDTA 0.1% (w/v)–ACN–methanol (MeOH) (1:1:1, v/v), acidified methanol 1% HCOOH and ethyl acetate-ACN-ammonium hydroxide (49:49:2, v/v) (Wang *et al*., 2021).

2.1.2. Solid-Phase Extraction

Solid Phase Extraction is sample Preparation Method used for isolation, enrichment and purification of components from aqueous solutions depending upon their physical and chemical properties (Patel *et al*., 2019). SPE is a fast and selective sample preparation and purification technique that is performed before chromatographic analysis. SPE technology allows sample purification, recovery, and concentration for precise quantitative analysis. The principle underlying the selectivity of SPE is similar to that of LC. SPE offers several significant advantages over LLE, such as less consumption of organic solvent, shorter analysis time, no phase emulsion, higher method recovery, and more efficient removal of interfering compounds (Wang *et al*., 2021). Also, the glassware used is expensive in liquid- liquid extraction (Patel *et al*., 2019).

Conventional SPE involves two essential steps: adsorption of the analytes onto the stationary phase and desorption from the solid material using small amounts of a favorable elution solvent (Chang *et al*., 2016). Common SPE cartridges and hybrid SPE cartridges are used to extract antimicrobials from chicken muscle (Wang *et al*., 2021).

SPE technology combines Ultra-Performance Liquid Chromatography (UPLC) with high-resolution mass spectrometry (HRMS) to quantitatively detect these analytes, and the Limit of Detection (LOD) (1 µg/kg) of these analytes are much lower than the value set by the EU. The development of this method has greatly improved the detection efficiency, and more than one hundred drugs can be measured simultaneously. The SPE method is widely used in the extraction of antimicrobials residues from chicken muscle. Efficient and simple extraction technology is conducive to the extraction of multiple residues. In addition, LLE and SPE are often used in combination to better enrich and purify antimicrobials in chicken muscle samples *(Wang et al*., 2021).

2.1.3. *Solid-Phase Micro-Extraction (SPME)*

SPME has been introduced as an alternative to traditional sample preparation techniques, because it provides a rapid, simple, effective, solvent-free, and sensitive pretreatment method and can also be easily combined with various separation techniques. The basic SPME device consists of a fused silica fiber (or a metal core) coated with an appropriate stationary phase. The fiber is fixed inside a needle of the syringe-like device. Extraction is performed either by immersing the fiber in the gaseous or relatively pure liquid medium, or by sampling the analytes from the headspace above the investigated medium. However, the use of SPME has some drawbacks. Mainly, commercially available SPME fibers are expensive and have limited lifetime, since they tend to degrade with increased usage. The difference in length and thickness of SPME fiber coatings may result in variation of analyte enrichment from fiber to fiber. These disadvantages limited its further applications (Chang *et al*., 2016).

2.1.4. *Matrix Solid-Phase Dispersion (MSPD) Extraction*

MSPD method is a rapid sample processing technique suitable for extracting antimicrobials residues from a single sample. Compared with modern extraction technology that uses high pressure and high temperature; ASE, MSPD performs the extraction process under ambient conditions and does not require any special laboratory equipment. It has advantages over conventional techniques, requiring only a few simple steps to extract a small number of samples and solvents. Based on these advantages, the MSPD method is widely used in the extraction of multiple antimicrobial residues from chicken muscle (Wang *et al*., 2021).

2.1.5. *Accelerated Solvent Extraction*

ASE is an automated method for extraction with organic solvents under conditions of elevated temperature and pressure. Introduced ASE as a new extraction procedure that uses organic solvents to extract solids or semi-solids at higher pressures (500–3000 psi) and higher temperatures (50–200 ◦C). The advantages of the ASE method are the small amounts of organic solvents, high speed, low matrix effect, high recovery rate and good reproducibility. The ASE method is widely used to extract antimicrobial residues from chicken muscle. The chicken muscle samples are placed into a mortar and added to diatomaceous earth for grinding. After being fully ground, the sample is filled into a 22 mL stainless steel extraction cell, and then the lid is closed. The cell is placed on the instrument, and the sample processing program is set (Wang *et al*., 2021).

Compared with LLE and SPE methods, ASE has the advantages of simple operation, high speed, and batch processing of samples, greatly improving efficiency and saving time. With the development of sample preparation technology, the automated ASE method is worthy of promotion for the extraction of antimicrobials residues from chicken muscle (Wang *et al*., 2021).

2.1.6. *Quick, Easy, Cheap, Effective, Rugged and Safe Extraction*

Modern sample preparation techniques focus on the reduction of organic solvent consumption in less time. These include QuEChERS methodology, Target analytes are extracted by acetonitrile, followed by an included liquid-liquid partition after the addition of salts and a dispersive solid-phase extraction clean-up step. This process was firstly developed for the extraction of pesticides from different matrices, but also for the extraction of drugs and phenols from soil and antimicrobials residues from chicken muscle (Marinou *et al*., 2019).

The steps of the QuEChERS method can be simply summarized as follows: (1) crushing of the sample; (2) single-solvent (acetonitrile) extraction and separation; (3) addition of MgSO4 and other salts to remove water; (4) addition of adsorbent to remove impurities; and (5) GC-MS and LC-MS analysis of the supernatant. The QuEChERS extraction method is widely used for multi-class or multi-residue analysis of different types of antimicrobials in chicken muscle. The principle of QuEChERS is similar to that of HPLC and SPE. It uses the interaction between the adsorbent filler and the impurities in the matrix to adsorb impurities, thereby achieving impurity removal and purification. QuEChERS method can extract both polar and non-polar compounds (Wang *et al*., 2021).

2.1.7. *Liquid Phase Micro-extraction (LPME)*

Traditional sample preparation techniques such as LLE have drawbacks in spite of the substantial use of this method over the years. The LLE method is tedious, time consuming and uses large amounts of toxic organic solvents which are non-compliant to the Green Analytical Chemistry (GAC) principles. In order to overcome these drawbacks, new extraction techniques that are simple, rapid and inexpensive, miniaturized and have the ability of automation have been developed in recent years. The efforts of various researchers in this area have resulted in the development of a new extraction technique known as liquid-phase micro-extraction. LPME offers an alternative to SPME. LPME can be divided into three main modes which are; single-drop liquid phase microextraction (SD-LPME), hollow fiber liquid phase microextraction (HFLPME) and dispersive liquid-liquid microextraction (DLLME) (Moyo & Tawanda, 2019).

Among these modes of LPME, HFLPME and DLLME have been the most used because of the advantages that they offer. SD-LPME is the least used mode because excessive stirring tends to break up the droplet, extraction is time consuming and reaching equilibrium can be a challenge. This disadvantage overrides the advantage that

this method has, which is the enormous reduction of volumes of organic solvent it uses. These methods are cheap and do not have sample carryover problems that are associated with SPME. LPME offers advantages such as high recovery and high enrichment factors, simplicity of operation, rapidity and they are also environment friendly (Moyo & Tawanda, 2019).

2.1.7.1. Dispersive Liquid-Liquid Micro-Extraction

DLME is a new LLE technique for the determination of poly-aromatic hydrocarbons and pesticides. The application of DLLME in the extraction of antimicrobials in literature has increased over the years. This technique is based on a ternary component solvent system including an extraction solvent, disperser solvent and an aqueous sample and is known as traditional DLLME. The advantages of traditional DLLME are the microliter-level volumes required for extraction and dispersive solvents and short extraction times. However, the disadvantage of traditional DLLME is the use of organic solvents as the extraction and dispersive solvents (Moyo & Tawanda, 2019).

Modified modes of DLLME have been invented recently and they include, low-density solvent based DLLME, solidified floating organic drop DLLME, effervescence assisted DLLME, air assisted dispersive liquid-liquid micro-extraction, surfactant assisted DLLME, cloud point DLLME, ionic liquid DLLME. Despite these disadvantages, DLLME is more advantageous in terms of short total time, low cost and feasibility compared with other liquid-phase micro-extraction techniques (Moyo & Tawanda, 2019).

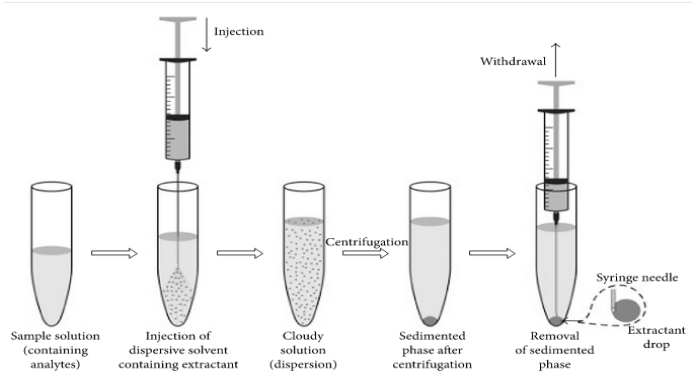

Figure 1. Schematic representation of DLLME technique
Source: Quigley *et al* (2016)

2.1.7.2. Hollow Fiber Liquid Phase Microextraction

Holow fiber liquid phase microextraction is a mode of LPME that uses a porous polypropylene hollow fiber for immobilization of organic solvent in its pores. The development of HFLPME provides a way to stabilize the extraction droplet in SD-LPME by placing it in a hollow fiber. The main consumable material is the hollow fiber membrane, which is lower than other methods in cost and sample consumption. The different modes of HFLPME are static, dynamic, two and three phase. The advantages of HFLPME are high enrichment, high degree of sample clean-up and low solvent consumption. The disadvantage of HFLPME procedure is that it is slow with extraction times ranging from 15 to 45 minutes and target analytes may partly be trapped in the supporting liquid membrane (SLM). Another disadvantage is that there is no complete setup commercially available for this method although hollow fibers are commercially available (Moyo & Tawanda, 2019).

2.2.Detection and Quantification of Antimicrobial Residues

There are So many sensitive and more specific methods were optimized and validated for qualitative and quantitative determination of different antimicrobials residues in chicken muscle samples such as Thin Layer Chromatography (TLC), ELISA, High-Performance Liquid Chromatography (HPLC), Liquid Chromatography (LC), Liquid Chromatography-Mass Spectrometry (LC-MS/MS) (Abd Elkhabeer *et al*., 2020). Capillary electrophoresis (CE), gas chromatography (GC), micellar electrokinetic capillary chromatography (MEKC), and advanced devices, including electrochemical biosensors, piezoelectric biosensors, optical biosensors, and MIP biosensors, are used to analyse antimicrobial in chicken muscle (Wang *et al*., 2021).

2.2.1. Screening/ Detection Test

Screening of food products from animal origin for the presence of antimicrobial residues started soon after the introduction of antibacterial therapy in veterinary medicine. Initially it mainly concerned process monitoring in the dairy industry to prevent problems in fermentative dairy production, but from the early 1970s regulatory residue screening in slaughter chicken muscle also became more commonly introduced. An efficient screening method needs to be low-cost and high-throughput, able to effectively identify potential noncompliant samples from a large set of negative samples (Ture *et al*., 2019).

Advantage of these methods is that they have a wide detection spectrum; they are simple to carry out and cheap; and can be used for the screening of a large number of samples; Possibility of automatization; Reduced time to obtain the result; Good sensitivity and specificity and Detection capability with an error probability < 5%. This method includes a large variety of detection methods, ranging from physico-chemical analysis or immunological detection to microbiological method (Ture *et al*., 2019).

2.2.1.1. Immunological Detection

Immunoassay is based on the specific reaction of antigens and antibodies including polymers-proteins, polypeptides, polysaccharides, and nucleoproteins. Numerous screening assays generally take a pretty long reaction time. So, there is a growing

need to introduce the immunoassays developed for the screening of antibiotic residues. Currently, the immunoassay of antibiotic residue detection is convenient, fast and some can achieve high throughput, while the methods have some of the limitations such as fewer numbers of drug detection, expensive instruments, time-consuming, and so on. Currently developed immune-based screening assays for the antimicrobials residue detection, such as ELISA, chemiluminescence immunoassay, radioimmunoassay, colloidal gold immunoassay and fluorescence immunoassay (Ahmed *et al*., 2020).

2.2.1.2. Enzyme-Linked Immunosorbent Assay

ELISA is a broadly documented method for the screening of antimicrobial residues. Regarding their high-throughput sample, these assays significantly increase numeral studies necessary to characterize the food samples for drug contamination (Ahmed *et al*., 2020). ELISA is an ultramicro-experimental detection technology with high sensitivity and specificity established by combining modern detection methods with immune technology. There are two ELISA formats: direct competitive-ELISA and indirect competitive-ELISA, of which the indirect competitive-ELISA method is more advanced (Wang *et al*., 2021). ELISA kits are allowing the analysis of a large number of samples per kit (Ture *et al*., 2019),

ELISA has the advantages of easy operation, convenience, high efficiency, strong specificity, and low detection cost and is widely used in the detection of antimicrobial residues in chicken muscle. The recovery and precision of the ELISA method are lower than those of the High-Performance Liquid Chromatography-Ultra Violet Detector (HPLC-UVD) method, but the sample preparation steps are simple, and the recovery and precision of the ELISA method meet the EU method parameter requirements (Wang *et al*., 2021). It has good performance for the analysis of antimicrobial residues in chicken muscle like tylosin and tetracycline, chloramphenicol, nitroimidazoles and sulphonamides and also for sedatives (Ture *et al*., 2019).

2.2.1.3. Microbiological Detection

Microbial inhibitions assays are very cost-effective and they have the potential to cover the entire antimicrobial spectrum within one test. There are two main test formats: the tube test and the (multi-) plate test. A tube (or vial, or ampoule) test consists of a growth medium inoculated with (spores of) a sensitive test bacterium, supplemented with a pH or redox indicator. At the appropriate temperature, the bacteria start to grow and produce acid, which will cause a color change. The presence of antimicrobial residues will prevent or delay bacterial growth, and thus is indicated by the absence or delay of the color change. This format is commonly applied in routine screening of milk, but it is also increasingly used for analysis of other matrices. In the plate test, the test sample is spread on the layer of the plate containing inoculated nutrient agar. Presence of an antimicrobial residue is detected by the formation of an opaque layer by the growing bacterial, thus yielding a clear growth-inhibited area around the sample if it contains antimicrobial substances (Ture *et al*., 2019).

2.2.1.4. Biosensors

Bioanalysis has been carried out by human beings forever, with the use of the nerve cells of the nose to detect scents or the enzymatic reactions on the tongue to taste food. With progress in understanding about the function of living organisms, scientific research has integrated them into man-made reactions to detect trace amounts of bio-chemicals in complex systems. Using bio-receptors from biological organisms or receptors, biosensors have been employed as a new mean of analytical and chemical analysis (Majdinasab *et al*., 2017).

According to The National Research Council (part of the US National Academy of Science), a biosensor is defined as a detection device that incorporates a living organism or product derived from a living system as the recognition element or a bio-receptor and a transducer to convert a biological reaction into a measurable signal or indication (Majdinasab *et al*., 2017). Compared to other detection techniques, biosensors are capable of performing residues screening relatively fast, accurately, and rapidly without the need for a specialist user. The selective and sensitive detection process of a biosensor depends on the most crucial component named biological recognition elements or bioreceptor whose signal is then detected with a suitable transducer. For fabricating a sensor, many bioreceptors such as enzyme, aptamer, antibody, DNA, microbial cell etc. have been most widely used (Khan, 2020).

For antimicrobials residue detection, the most frequently used biosensors are those based on antibody/antigen affinity pairs, which are widely used in the immunochemical screening of samples (Majdinasab *et al*., 2017).

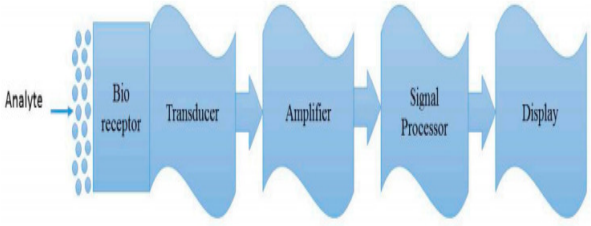

Figure 2: Schematic representative diagram of a biosensor.

Source: Majdinasab *et al*. (2017)

Different types of biosensors have been developed in recent years as an alternative approach to screen antimicrobials in chicken muscle (Ture *et al*., 2019); including methods involving electrochemical biosensors, piezoelectric biosensors, optical biosensors, and MIP biosensors. The developed sensor analysis method not only has the advantages of simple operation, high speed

and low cost but also provides satisfactory results in terms of specificity, sensitivity, and recovery in the detection of antimicrobials in animal-derived foods (Wang *et al*., 2021).

In general, these sensors usually contain an antibody as a recognition element that interacts with the analyte; the resulting biochemical signal is measured optically or converted into an electronic signal that is further processed in appropriate equipment. Biosensors can be able to detect simultaneously multiple antimicrobial residues in a sample at a time. In general, these sensors are valid for control laboratories because they can detect multiple residues in one sample and can thus allow the analysis of a large number of residues and samples (Ture *et al*., 2019).

2.2.2. Identification and Confirmation

Nowadays, different analytical and screening methods are available. Chromatographic analysis like thin layer chromatography, liquid chromatography, gas chromatography and more advanced mass spectrometry have been replacing the conventional microbiological and immunological detection and quantification of antimicrobials residue from chicken muscle samples as these methods provide more recovery rate even several-fold higher. Hence, chromatographic techniques are preferred to other analytical techniques even though they are quite expensive and sophisticated (Nazmul *et al*., 2021).

Different analytical techniques are available for the purpose of antimicrobials residues identification and confirmation from chicken muscle. When the target analyte is clearly identified and quantified above the decision limit for a forbidden substance or exceeding the maximum residue limit (MRL) in the case of substances having a MRL, the sample is considered as non-compliant (unfit for human consumption). Identification is easier for a limited number of target analytes and matrices of constant composition. Some examples of the available confirmatory methodologies are as follows (Ture *et al*., 2019).

2.2.2.1. Thin-Layer Chromatography

TLC is a sensitive and exact-reliable method for monitoring low amount of different biological and chemicals. TLC is among the most helpful instruments for following the advancement of organic chemical reactions and for measuring the pureness of organic compounds in phytochemistry and Biotechnology. Like every single chromatographic methods, TLC exploits the distinctive affinity of the analyte with the mobile and stationary phases to accomplish the partition of complex mixtures of organic molecules. Illumination of antibiotics against UV light helps as a simple detector for this mean. Employment of TLC to pharmaceutical and medical or clinical or biological research comprises more than 50% of the technique's total application. An important application of TLC is determination of drug residues in food (Nazmul *et al*., 2021).

Advanced one; High Performance Thin-Layer Chromatography (HPTLC) allows the qualitative and quantitative detection of antimicrobial residues in chicken muscle but its use has rapidly decreased due to the expansion of other techniques like HPLC. Reported uses of HPTLC applied to meat include the detection of residues like clenbuterol and other agonists; nitroimidazol, sulphonamides and thyreostatic drugs. The plates are sprayed with an appropriate chromogenic reagent or viewed under UV light for visualisation of compounds. Detection by fluorescence is also applied. Quantitation is achieved by measuring the relative intensity of the spot vs that of the internal standard by scanning densitometry. Modern HPTLC has been automatized at a high level (Shankar *et al*., 2010).

2.2.2.2. Liquid Chromatography

LC is a common, efficient, and rapid chromatographic method to detect antimcrobials in chicken muscle. The key to LC separation is to select a suitable chromatographic column and optimize the composition of the mobile phase and the elution procedure. The LC method has wide applicability and can be used for most antimicrobials. Generally, analyses of antimicrobials are conducted by LC coupled with specific detectors, such as fluorescence detectors, diode array detectors, ultraviolet detectors and evaporative light scattering detectors. At present, the pairing of LC with mass spectrometer detectors (MS and tandem MS) has been widely used in the analysis of antimicrobials in chicken muscle (Wang *et al*., 2021).

Different types of detectors combined with LC are used to detect the same type or different types of antimicrobials and have their own advantages and disadvantages. FLDs are highly sensitive and selective detectors that can detect only compounds that produce fluorescence. DADs and UVDs are mainly used to detect antimicrobials containing ultraviolet absorbing groups, and they have the advantages of high sensitivity, low noise and wide linear range. ELSDs can detect any sample with lower volatility than the mobile phase. Due to this limitation, it has rarely been used in the detection of antimicrobials residues in chicken muscle in the past decade *(Wang et al*., 2021).

Compared with FLDs, DADs, UVDs and ELSDs, MS detectors can simultaneously detect more than 100 antimicrobials in chicken muscle. In addition, MS detectors have high recovery, high selectivity, good reproducibility, and low interference. Moreover, the use of tandem MS improves sensitivity and plays an important role in confirming false positives. The rapid development of MS detectors, such as triple quadrupole-MS, time of flight (TOF)-MS, quadrupole (Q)-TOF-MS and Orbitrap-HRMS instruments, has greatly improved the efficiency of detecting antimicrobials in chicken muscle. The LC-MS/MS method is commonly used to detect antimicrobials residues in chicken muscle. (Wang *et al*., 2021)

2.2.2.3. *Liquid Chromatography-Mass Spectrometry/ Mass Spectrometry*

LC-MS is an alternative technical approach that is now popular for the screening of more than a hundred antimicrobials in a single run. By the end of the 20th century, LC-MS had evolved dramatically as a major analytical tool, providing both sufficient sensitivity to reach the regulatory limits and adequate certainty in the identification of the compounds detected. Furthermore, LC-MS is versatile enough to be used either as a screening tool or a quantitative method (or both), depending on the application (Delatour *et al*., 2018).

Since the last decade liquid chromatography coupled to mass spectrometry has become an essential technique in food analysis laboratories. Its main application is related to the determination and confirmation of residues of contaminants, and usually the existing methodologies allow the analysis of a series of compounds belonging to the same family, i.e. compounds having similar physicochemical properties. Nowadays an effort is being made to develop multiclass methods for antimicrobials. The main difficulties encountered during the analytes extraction and clean-up of the extracts. Thus, the extraction solvent must be selected on the basis of the properties of the different groups of target compounds and taking into account that acceptable recoveries should be obtained for the whole set of substances. On the other hand, clean-up optimization can be complex, particularly if a relatively large number of families are considered (Chico *et al*., 2008).

2.2.2.4. *Gas Chromatography*

GC is a commonly used chromatographic technique that mainly uses differences in the boiling point, polarity, and adsorption properties of compounds to separate mixtures. For the analysis of antimicrobials residues in chicken muscle, GC instruments are usually connected to classic detectors, mainly including nitrogen-phosphorus detectors (NPDs), electron capture detectors (ECDs) and MS detectors. To date, GC-MS and GC-MS/MS methods are the most commonly used methods to detect antimicrobials in chicken muscle. Compared with NPDs and ECDs, MS or MS/MS has good recovery, precision, and reproducibility and can confirm false positives. Generally, derivatization reactions are required for the detection of antimicrobials by GC. GC usually requires the selection of specific capillary columns to separate the antimicrobials in the sample, while optimization of the mobile phase, as in the LC method, is not required (Wang *et al*., 2021).

GC instruments are relatively expensive, and researchers usually need professional training to operate the instruments. GC is widely used for analysis of pesticides, and GC-MS/MS methods are being gradually developed for research on antimicrobials. The main reason is that mass spectrum information for some antimicrobials in the GC mass spectrum library is lacking (Wang *et al*., 2021).

2.2.2.5. *Capillary Electrophoresis*

CE is a new type of liquid-phase separation technology that uses capillaries as the separation channel and a high-voltage direct current electric field as the driving force. CE is an efficient, fast, and economical automated separation technology with the advantages of low reagent consumption, small sample injection volume and high separation efficiency. CE has certain limitations in sample preparation ability, sensitivity, and separation reproducibility. The reasons for these limitations are the small injection volume, small capillary diameter, and electroosmotic changes due to sample composition. Due to the low sensitivity caused by the small injection volume, CE has been combined with some high-sensitivity detectors, including UVDs, laser-induced fluorescence detectors, Electro-Chemi-Luminescence detectors, DADs, MS detectors and chemi-luminescence (CL) detectors. (Wang *et al*., 2021)

2.2.2.6. *Micellar Electro-Kinetic Capillary Chromatography*

MEKC is a novel hybrid method that combines the separation principles of chromatography and electrophoresis. MEKC is an important form of CE and has become one of the most popular technologies for separating antimicrobials due to its high separation power and ability to separate both ionic and neutral compounds. In MEKC, an ionic surfactant such as sodium dodecyl sulfate is added to the buffer to form micelles. The separated substances are distributed between the aqueous phase and the micellar phase and migrate in the capillary with electroosmotic flow, thereby achieving a separation effect. Because of its simple operation, few sample pre-processing steps, and low instrumentation cost, this method was used in the detection of antimicrobials residues in chicken muscle in recent years. (Wang *et al*., 2021)

2.2.2.7. *UPLC-MS/MS*

Over the past decade, food safety has become an important issue worldwide due to higher incidences of food contamination. Currently, one of the great challenges in food safety is the analysis of emerging food contaminants like, drug residues. Moreover, the scope, relevance, and level of food safety and testing have never been in such complexity than in today's global marketplace. In recent years, a novel technology ultra-performance liquid chromatography coupled with MS has been developed to estimate drug residues as well as other food contaminants from chicken muscle with better accuracy, sensitivity, precision, and high throughput (Syed *et al*., 2020).

UPLC is a novel technique that offers a new pathway for LC. UPLC enhances the capability of LC in four main areas like increasing speed, sensitivity, resolution and accuracy. UPLC is also known as Ultra-High-Performance Liquid Chromatography. In comparison to HPLC, UPLC has been upgraded with column

packing materials of less than 2 µm in diameter, which increases the speed, accuracy, resolution and sensitivity. Moreover, particle size used in HPLC, UPLC column ranges from 3 to 5 µm and < 2 µm respectively as well as mobile phase flow rate in HPLC is usually 3.0 ml/min compared to UPLC flow rate 0.6 ml/min. The basic difference in the principle of UPLC and HPLC is the column packing material, which makes a huge difference over the sensitivity and accuracy of the novel techniques. Apart from the principle involved in the LC, there is not much change in basic principle except the pressure generated or created in the instruments make it a more efficient technology. Efficiency of this technique is equivalent to the dimension of the column and inversely proportional to the radius of the atoms. As the name suggest ultra-performance or ultra-pressure, UPLC works under very high pressure up to 1000 bars, however for HPLC, pump pressure not go more than 300–400 bars (Syed *et al*., 2020).

In recent years, the demand of UPLC-MS/MS in food analysis has increased, because of the novel characteristics of UPLC with good resolution, better accuracy and sensitivity and reproducibility. Since its inception, it has reduces the time of food scientists as well as cost of the analysis because of its capability of producing more valuable, reliable, and reproducible data. The UPLC sensitivity has reached to ppb and ppt levels by virtue of which a food analyst would be more confident in ensuring safe food for consumption. Analysis of several antimicrobials from chicken muscle samples has been performed using UPLC-MS/MS technique. UPLC–MS works on van Deemter principle, which states that, "the flow rate of smaller particles are much faster in compare with large particles as well as unfolding the correlation of flow rate and plate height." Various food components as well as food contaminants such as vitamins, amino acid, metabolite identification, adulteration, forensic testing, toxicity studies, phytoconstituents, pesticide in agriculture, antimicrobial residue, hormones, dyes and pigment analysis can be performed using UPLC–MS (Syed *et al*., 2020).

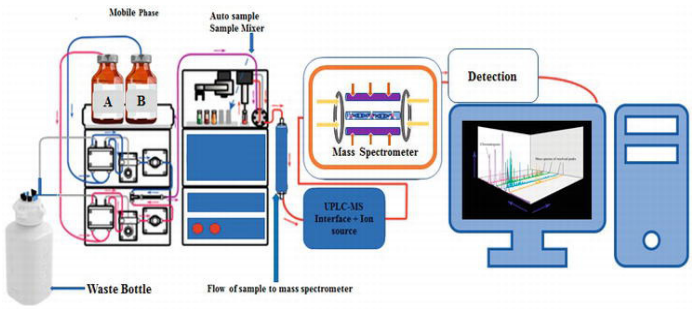

Figure 3: Flow diagram representative of ultra-performance liquid chromatography-mass spectrometry

3. Maximum Residue Limit of Antimicrobials in Chicken Muscle

Globally, more than half of all medicines are prescribed, dispensed or sold improperly. This is more wasteful, expensive and dangerous, both to the health of the individual patient and to the population as a whole that magnifies the problem of misuse of antimicrobial agents. In many African countries, antimicrobials may be used indiscriminately for the treatment of bacterial diseases or they may be used as feed additives for chicken. The ongoing threat of antimicrobials contamination is one of the biggest challenges to public health that is faced by the human population worldwide. Such residues are spreading rapidly, irrespective of geographical, economical, or legal differences between countries (Ture *et al*., 2019).

Some countries have their own MRL and some follow World health organization/Food and drug authority (WHO/FDA). Ethiopia does not have its own MRL and there is also no information on that the country adapted

MRL from others (Mamo, 2018).

The European Union has strictly regulated the use of antimicrobials in chicken muscle. Some of these drugs can be permitted only in specific circumstances (therapeutic purposes) but under strict control and administration by a veterinarian. The use of substances having hormonal or thyreostatic action as well as β-agonists is controlled by official inspection and analytical services following Commission Directive 96/23/EC on measures to monitor certain substances and residues in chicken muscle. Establishment of a legislative framework and of an institutional structure is the first step in the assessment and management of drug-related risk. From this point of view, according to pending European legislation the use of antimicrobials must be based on risk evaluation. The risk due to the use of antimicrobial is "any risk for animal or public health relating to the quality, safety and efficacy of antimicrobials and any risk of undesirable effect on the environment". Risk management is a task of both private and public veterinary services that are involved in the prevention and control of all hazards arising from the use of antimicrobials (Ture *et al*., 2019)

In all cases, the levels of residues chicken muscle should not harm the consumer. In table 1, the codex committee determines residues of antimicrobials in chicken muscle and recommended MRLs for antimicrobials. A codex maximum limit for residues of antimicrobials is the maximum concentration or residue that results from the use of an antimicrobials (expressed in mg/kg or g/kg on a fresh weight basis) recommended by the CAC to be legally permitted or recognized as acceptable in or on a food.

Table 1: CAC Maximum Residue Limits for Residues of antimicrobials in Chicken Muscle

Source: Codex Alimentarius (2018)

MRL is based on the type and amount of residue considered to be without any toxicological hazard from human health as expressed by the acceptable daily intake, or on the basis of a temporary ADI that utilizes an additional safety factor. MRL also considers public health risks as well as food technology issues. Acceptable daily intake is an estimate by the joint FAO/WHO expert committee on food additives (JECFA) of the amount of an antimicrobials, expressed on a body weight basis, which can be ingested daily over a lifetime without appreciable health risk which is put in table 1 above. When establishing a MRL, residues that occur in food of plant origin and/or the environment are also considered. Furthermore, a MRL may be reduced to be consistent with good practices in the use of antimicrobial to the extent that practical and analytical methods are available.

4. DISCUSSION

When looking some assessment/researches on the antimicrobials residues in chicken muscle samples; Adla Jammoul and Nada El Darra (2019) reported that "from 80 tested samples 22.5% of samples are none detected and from those samples 77.5% of samples were contaminated at least with one antimicrobial residue. Out of the contaminated samples, 23.75% were contaminated with one antimicrobial residue and 53.75% were contaminated with more than one antimicrobial residue. The frequency of detection of the three antimicrobial agents in chicken muscle from which the frequency of detection of tetracycline was 18.1% (21 of 116). β-lactam was detected at a frequency of 1.7% (2 of 121). For macrolides, the residue was 0.0% (0 of 123). In this research Overall frequency of detection of antimicrobial agents (tetracycline, β-lactam and macrolides) in chicken muscle has been reported as 6.4% from 116 chicken meat samples. Another study by Seyda S. *et al*. (2018) reported that, "residues of enrofloxacin, doxycycline and tylosin from a total of 300 analyzed samples (whole chicken, drumstick and breast meat) 11 (3.6%) were found. Six (2%) of these 11 samples had enrofloxacin, 3 (1%) had doxycycline and 2 (0.6%) had tylosin residues. Since the results of this study showed that the majority of samples were lower than allowed limits."

Al-Ebrahim & El-Ghareeb (2020) also reported, "A total of 120 broiler meat samples (60 breast samples and 60 thigh samples) were examined using LC-MS/MS for determination of fluoroquinolones residues (sarafloxacin, danofloxacin, ciprofloxacin, enrofloxacin, ofloxacin, and marbofloxacin) at Al-Ahsa province. Incidence of fluoroquinolones residues in examined breast meat samples were 44 (73.33%) for sarafloxacin, 45 (75%) for danofloxacin, 49 (81.67%) for ciprofloxacin, 48 (80%) for enrofloxacin, 50 (83.33%) for ofloxacin, 45 (75%) for marbofloxacin while in thigh muscles were 49 (81.67%), 45 (75%), 49 (81.67%), 48 (80%), 55 (91.67%) and 45 (45%) for sarafloxacin, danofloxacin, ciprofloxacin, enrofloxacin, ofloxacin and marbofloxacin, respectively. All examined samples were within permissible limit except for enrofloxacin (one breast sample) and sarafloxacin (20 and 14 breast and thigh samples, respectively) were higher than permissible limits." Al-Ebrahim & El-Ghareeb (2020) reported 48 (80%) residue detected out of 120 breast and thigh meat samples for enrofloxacin is greater than that of 3 (1%) residue out of 300 fresh and packaged chicken meat samples reported by Seyda S. *et al*., 2018. Mamo S. (2018) reported, "A total of 90 samples (thirty breast meat samples, thirty leg meat samples and thirty gizzard meat samples) were analyzed for the determination of oxytetracycline in Bishoftu town, Ethiopia. The result obtained from analysis shows that all the chicken meat samples (90 meat samples from the 30 chickens) were at non-detectable level of oxytetracycline residue." Mamo S. reported oxytetracycline residue is much smaller than the result reported by Adla Jammoul and Nada El Darra (2019) in Lebanon which was 18.1% (21 of 116).

Drugs	Food commodity		MRL
	Species	Tissue	µg/kg
Avilamycin	Chicken	Muscle	200
Benzylpenicillin/ procainebenzylpenicillin	□	□	50
Chlortetracycline/ oxytetracycline/ tetracycline	□	□	200
Colistin	□	□	150
Danofloxacin	□	□	200
Diclazuril	Poultry	□	500
Dihydrostreptomycin/ streptomycin	Chicken	□	600
Erythromycin	□	□	100
Flumequine	□	□	500
Lincomycin	□	□	200
Monensin	□	□	10
Narasin	□	□	15
Neomycin	□	□	500
Nicarbazin	□	□	200
Sarafloxacin	□	□	10
Spectinomycin	□	□	500
Spiramycin	□	□	200
Sulfadimidine	Not specified	□	100
Tilmicosin	Chicken	□	150
Tylosin	□	□	100

5. Conclusion and Recommendations

5.1. Conclusion

Presence of antimicrobials residues in chicken muscle above the maximum levels or residue limits is recognized worldwide as an important food safety and human health hazards. Drug residues are now monitored worldwide by many government and private contract laboratories to enforce regulations domestically and in international food trade. The presence of antimicrobial residues in chicken muscle, is due to failure to comply with the instructions for their use or poor livestock production practices. Chicken muscle

contaminated with drug residues may pose serious public health hazards, affect economic sector of the world as well the environment. The limitation of codex and world trade organization to enforce adoption of MRLs, has resulted in differences in food safety standards across countries and nations. Such differences usually end as trade disputes leading to a gradual decline in meat and meat products exported.

Some countries have their own MRL and some follow world health organization/food and drug authority. Ethiopia does not have its own MRL and there is also no information on that the country adapted MRL from others. Animal-derived food samples have a complex matrix and many endogenous interfering substances, making it impossible to directly detect drug residues. Reliable sample extraction methods for detection of these residues are necessary to ensure food safety. There are so many sensitive and more specific methods were optimized and validated for qualitative and quantitative determination of different antimicrobials residues in chicken muscle samples. Antimicrobial residues prevalence in chicken muscle samples have different values in different countries. In Ethiopia antimicrobials residues prevalence in chicken muscle samples are at non-detectable level.

5.2. Recommendations

- To know antimicrobial residue in chicken musclefurther investigation is required by using advanced residue analytical methods and equipment
- To control and regulate drug residue risks from chicken muscle needs supporting and advancing of drug residue analysis centers
- Setting up policy and regulation is required; adapting MRL from FDA or WHO to monitor and control antimicrobials residues in chicken muscle
- Further assessment/research of drug residues in chicken muscle using modern analytical methods and equipment

LIST OF ABBREVIATIONS

ADI	Acceptable daily intake
ASE	Accelerated solvent extraction
CE	Capillary electrophoresis
CAC	Codex alimentarius commission
DLLME	Dispersive liquid-liquid microextration
ECD	Electron capture detector
EDTA	Ethylene diamine tetraacetic acid disodium salt
GC	Gas chromatography
HPLC	High performance liquid chromatography
UVD	Ultra-violet detector
HPTLC	High performance thin layer chromatography
HRMS	High resolution mass spectrometry
HFLPM	Hollow fiber – liquid phase microextraction
JECFA	Joint Expert Committee on Food Additives
JMPR	Joint Expert Meeting on Pesticide Residues
LC	Liquid chromatography
LLE	Liquid-liquid extraction
MSPD	Matrix solid phase dispersive
MRL	Maximum residue limit
MEKC	Micellar electrokenetic capillary chromatography
MIC	Minimum inhibitory concentration
MIP	Molecularly imprinted polymers
NPD	Nitrogen phosphate detector
NOAEL	No-observed-adverse-effect level
QUECHERS	Quick, easy, cheap, effective, rugged and safe
SPE	Solid- phase extraction
SPME	Solid phase micro-extraction
TLC	Thin layer chromatography
UPLC	Ultra-performance liquid chromatography

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