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LIST OF ABBREVIATIONS

ADI	Acceptable Daily Intake
AFSSA	Agence Franaise de Securite Sanitaire des Aliments
AVMA	American Veterinary Medical Association
BKT	Belgian Kidney Test
CAF	Chloramphenicol
CAST	Calf Antibiotic and Sulfa Test
CSA	Central Statistical Authority
DDT	Dichloro-Diphenyl-Trichloroethane
EC	European Commission
EDTA	Ethylenediaminetetraacetic acid
EEC	European Economic Commission
ELISA	Enzyme-linked Immunosorbent Assay
EMEA	European Agency for Evaluation of Medical Products
FAO	Food and Agriculture Organization of the United Nations
FAST	Fast Antibiotic Screen Test
FPT	Four Plate Test
FSIS	Food Safety and Inspection Service
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
IDF	International Dairy Federation
Ig	Immunoglobulin
IPCS	International Programme on Chemical Safety
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LAST	Live Animal Swab Test
LC	Liquid Chromatography
MRL	Maximum Residue Limit
NMPF	National Milk Producers Federation
NMSA	National Metrological Center Agency
NOEL	No Observed (adverse) Effect Level

NSAIDS	Non Steroidal Anti-inflammatory Drugs
OIE	Office International des Epizooties
STOP	Swab Test On Premises
TEAGASC	Irish Agriculture and Food development Authority
TLC	Thin Layer Chromatography
USDA	United State Department of Agriculture
USFDA	United States Food and Drug Administration
UV	Ultraviolet
WHO	World Health Organization

ABSTRACT

A cross-sectional study was conducted from October 2006 to May 2007 to investigate the proportion of tetracycline residue levels in beef at Addis Ababa, Debre Zeit and Nazareth slaughterhouses. A total of 384 muscle and kidney samples were randomly collected from slaughtered cattle in the respective slaughterhouses. The samples were qualitatively screened for tetracycline residues by thin layer chromatography using a suitable silica gel (Merck silica gel 60 plates).The positive samples were then further analyzed by using high performance liquid chromatography (HPLC) Shimadzu Model Class-VP series with an electron diode array detector. The result of this study indicated that oxytetracycline residues previously detected in thin layer chromatography were detected in all samples by HPLC. A given sample was regarded as positive for tetracycline if its retention time and peak corresponded to that of the standard. Out of the 384 samples that were analyzed for tetracycline residues 274 (71.35%) had detectable oxytetracycline residues. Tetracycline and doxycycline were not detected. At Addis Ababa slaughterhouse, 120 (93.75%) of kidney and beef samples were positive for oxytetracycline. In Debre Zeit 48 (37.5%) of kidney and beef samples were also positive for oxytetracycline while from Nazareth slaughterhouse 106 (82.81%) of kidney and beef were positive for oxytetracycline. The mean ($p>0.05$) residue levels of oxytetracycline for the three slaughterhouses studied in muscle were as follows: Addis Ababa 108.34µg/kg, Nazareth 64.85µg/kg and 15.916µg/kg at Debre Zeit while in kidney samples were ($p<0.05$): 99.02 µg/kg in Addis Ababa, 109.35µg/kg in Nazareth and 112.53µg/kg in Debre Zeit slaughterhouses. The oxytetracycline positive samples, which showed residues of oxytetracycline above maximum residue limits (100µg/kg) in muscle samples, were 58 (48.33%) at Addis Ababa slaughterhouse and 51 (48.11%) at Nazareth slaughterhouse and 1 (0.9%) in the kidney samples of Nazareth slaughterhouse. At Debre Zeit slaughterhouse no samples were above the maximum residue limit. The results obtained confirmed that oxytetracycline was heavily and indiscriminately used in the fattening farms, small scale fattening farms, pastoralists and other animal owners. The results also suggest that the recommended withdrawal time was not strictly applied. Results of the present study could serve as a preliminary base line information for veterinary authorities, drug administration and quality control authority, other concerned organizations and professionals to take measures on control

and prevention of drug residues; as well as the consumer to be aware of the possible drug residue risks through consumption of unsafe animal products.

Key words: Tetracycline, residue, beef, kidney, Addis Ababa, Debre Zeit, Nazareth slaughterhouses

1. INTRODUCTION

The growing challenge to secure wholesome food of animal origin in quantities sufficient to feed the ever increasing world population leads to the compelling need to search for new means of enhancing productivity in animal husbandry. However, such an endeavor often involves the use of physiologically potent substances (FAO, 1984).

In countries with large-scale animal production, a percentage of animals are exposed at one time or another during their life-span to various chemicals, such as drugs to prevent or cure disease and feed additives to increase feed efficiency and to promote growth (FAO, 1984). As agricultural production is increasingly becoming an industrial operation all over the world, the trend of introducing further new substances will certainly continue. Even by contemporary production practices, many drugs are administered to increasingly large herds and flocks during substantial portion of the life-span of animals (eg. coccidiostats) or, for example at the height of milk production (eg. fasciolicides, oestrus-syncronizating agents) or shortly after slaughter to reduce the effect of stress (eg. tranquilizers and beta adrenogenic blocking agents). Hence, at some levels, their residue will reach the consumers via food of animal origin. In recent years, public concern over the presence of drug residue in edible products of food producing animals has rapidly grown and still growing. Consequently, in many countries legislation regulating the use of drugs in animal has been instituted with the primary aim to avoid their residues in food (FAO, 1984).

A residue is defined in EC directive 96/2B/EC as a residue of substances having a pharmacological action, of their metabolites and of other substances transmitted to animal products and which are likely to be harmful to human health. Almost all chemicals administered knowingly or unknowingly to animals result in some trace residue remaining in the milk, meat or other animal products. It is also very important to differentiate between safe and unsafe levels of drug residue rather than to insist on zero (0) residual concentrations. No chemical is safe under all conditions of use. It is therefore important that all are fully evaluated for safety, as the parent compound and/or as its metabolites and that the results of these evaluation determine acceptability. The maximum residue limit is the maximum concentration of residue resulting

from the use of a veterinary medicinal product which may be legally permitted or recognized as acceptable in or on a food, allocated to individual food commodities (Gracey *et al.,* 1999).

The therapeutic products that cause concern fall into a number of categories. The major once are antimicrobials, which are a diffuse group containing several classes of compounds used to treat or prevent bacterial infection. Approximately 42% of all veterinary pharmaceuticals used worldwide are used as feed additives,19% are used as anti-infective (e.g., antibacterials and antivirals),13% as parasiticides,11% are used as biologicals and 15% represent other pharmaceuticals. In volume and money value, antimicrobials represent the largest proportion of pharmaceutical sales of drugs used in animal production. (Miller,1993). Twenty-five million pounds of antibiotics are fed to American livestock annually. This is about 70% of the total amount of antibiotics produced in the U.S. each year and eight times more than the amount used as human medicine (Union of Concerned Scientists, 2001). Scientists do not understand how or why the drugs promote growth. Many of the same antibiotics, six of the 17 classes of antibiotics used to promote growth in animals are also used to treat diseases in humans (The New York Times, 1999). The pesticides are also diffuse group including anthelmintics used for their activities against round worm, tape worms and fluke, ectoparasiticides used to kill external parasites and anti protozoan. Hormones are used for therapeutic in various fertility treatments or for growth promotion and are administered as injection or implant. Animals are also exposed to many environmental contaminants including herbicides, heavy metals and fungicides. Some of these substances find their way into animal's tissue via feed (Gracey *et al.*,1999).

The earliest screening methods used for detecting antimicrobial residues in foods, including milk, were based on the detection of growth inhibition of various bacterial strains. Such methods were based on microbial agar diffusion tests or on the inhibition of acid production by starter organisms (Mitchell *et al*., 1998). From the 1950s, assays were developed for testing of tissues primarily from the existing milk testing procedures. In 1970 antimicrobial test for both detection and identification of antimicrobial in meat was described (VanDresser and Wilcke, 1989).

In developing countries, where no regulatory legislation or mechanisms are in place for drug approval and use or for residues monitoring the occurrence of residues is not surprising. Drugs,

including unapproved and unregulated compounds that may have no acceptable daily intake, can often be easily purchased from village shops and informal vendors. There may be no quality control of the substances supplied and this may be compounded by illiteracy, rendering, labeling and printed instructions for drugs of limited use. Drugs are frequently administered by unqualified individuals or para-veterinary field staff and extended usage or excessive or multiple dosage of the compounds are common. Treatment records are frequently poorly maintained or non-existing and individual animal identification and traceability is often impossible. Due to a scarcity of meat and fear of economic loss usually poor farmers slaughter casaulity animals for food (Buzalski *et al*., 2004).

Protection of the public health against harmful effects of veterinary drug residues is relatively recent preoccupation. The initial intention for adequate consumer protection led to the desire to archive complete elimination of all traces of drug residues in food commodities. Therefore, animal drugs were initially approved based on a "no residue" tolerance policy, but actually the "zero" tolerance represented the sensitivity of the analytical method to monitor for drug residues. As analytical methods improved, the "no residue" tolerance was continually being lowered. Ultimately, a policy of negligible tolerance, based on toxicology data was developed (Boisseau, 1993; Teske, 1993).

In Ethiopia, there is low level of control from the government authorities and information on the actual rational drug use pertaining to veterinary drug use is not available so far which are essential for risk analysis and planning. Besides these, misuses of drugs are highly practiced among these tetracycline is one of the commonest drug. It is common to see drugs sold in the market and along the roads by informal vendors for administration by farmers themselves and no record keeping at all. With regard to public health, the consumers incur adverse consequences of antibiotic residue in food. There is lack of awareness and preparedness among the controlling authorities to deal with the risk of antibiotic residues to the consumers. In addition, there is lack of comprehensive studies on drug residues in the country. Furthermore, animals being slaughtered in the country are not screened for the presence of residue in any of the slaughterhouses in the country so that people could chose or buy meat products with out antibiotic residue. No Controlling authorities exist in the country to protect the consumers against

the consumption of meat and milk products containing harmful drug residues. Therefore, drug residues should be one of the most emerging food hygiene and safety issues which require immediate attention by the controlling authorities and concerned organizations. Thus, the present study was formulated with the following objectives:

- To detect tetracycline residues in beef and kidney samples from slaughtered cattle at Addis Ababa, Debre Zeit and Nazareth public slaughterhouses.
- To quantitatively analyze tetracycline residue levels in those muscle and kidney samples that had showed positive results by qualitative analysis using thin layer chromatography (TLC)
- To adopt methods for identification of antimicrobial residues in meat.

2. LITRATURE REVIEW

2.1. Source of drug residues

Several types of residue are found in food animals. Industrial, chemicals, agricultural chemicals, food additives, and veterinary therapeutic agents. The food additives and therapeutic agents are of constant concern to veterinarians involved in the production and processing of food animals (Hagstad and Hubbert, 1989).

2.1.1. Antimicrobials

The most likely cause of violative drug residues is the failure to observe withdrawal times (Paige and Kent, 1987; VanDresser and Wilcke, 1989; Guest and Paige, 1991; Paige, 1994). Improper maintenance of treatment records or failure to identify treated animals adequately may lead to their omission (Sundlof, 1990).Violative drug residues can also occur as a result of improper use of licensed product through the illegal use of unlicensed substances. Extra label dosages and use of drugs which have not been approved for the species in question may lead to violative residues (Papich *et al.,*1993; Kanneen and Miller, 1997; Higgins *et al*.,1999).

The disease status of an animal and the way in which drugs are administered influence the potential for residues. Disease may affect the pharmacokinetics of the drug, metabolism, or the presence of infection and/or inflammation may cause the drug to accumulate in affected tissues (Kanneene and Miller, 1997). Contamination of animal feeding stuffs with a variety of compounds also occurs. The significance of this contamination depends on the pharmacodynamics of the compound and the species affected (McEvoy, 2002). Indeed, contamination of feeding stuffs seems to be an important source of contaminated application of antimicrobials.

2.1.2. Growth promoters (hormones)

Growth promoting hormones are other sources of residue in food of animal origin (Gracey *et al*., 1999). Some of these are:

- Natural sex steroid hormones – oestradiol, progesterone, testosterone

- Synthetic-steroid androgens- nanrolone, norethnrolone, nortestestrone, phnylpropionate, ethinylosteradiol and laurate.
- Synthetic nonsteroidal oestrogens-stilbeneoestrogens (diethylstilboestral (des), hexoestrol), zeranol and trenboloneacetate.
- Peptide hormones- growth hormone (Gh), growth hormone releasing factors and thyotrophin-releasing hormone (Thr).
- ß-adrenocepter agonist (beta-agonist)-clenbuterol and cimateratol.

2.1.3. Pesticides

Pest control chemicals must be toxic to some living organisms to fulfill their role depending on the pest being controlled they may be termed insecticides, fungicides, etc. The insecticides that are directly applied to food animals and anthelmintics are regarded as the most important subgroups (Gracey *et al*., 1999).

2.1.4. Insecticides

The chlorinate hydrocarbons (e.g. DDT) are extremely durable, persistent and bio-accumulating compounds which find their way into the feed chain usually through use in controlling environmental or animal pests. The more recently developed organophosphate insecticides are excreted rapidly and do not persist to the same extent in the environment, however, frequently they are more toxic in small amounts as their biological activity is greater. The organophosphates (eg. coumaphos, malhation, dichlorphos, diazinon) are extremely toxic to mammals, but are highly efficient insecticides (Gracey *et al*., 1999).

2.1.5. Heavy metals

Excessive intakes of heavy metals in food have caused intoxication in man. These are more often caused by contaminated cereals or by accidental additions during processing occasionally toxic concentrations occur in animal tissues and products. These can be associated with soils naturally high in the associated element through environmental contamination from local industry and are cumulative in animal tissues. They may also occur from feeding grain treated with toxic metals or

from excess amounts remaining in the environment following previously used paints etc. Some of these metals are: lead, arsenic, mercury, cadmium, copper and selenium (Gracey *et al.,* 1999).

2.1.6. Other substances

Fluorine, NSAIDS, especially phenylbutazone ("bute"), natural toxins (mycotoxins, shell fish) do also have residual effect which can be dangerous to the human health (Gracey *et al*., 1999).

2.2. Use of veterinary drugs

Drugs in food animals can be used as therapeutic, prophylactic and growth promotion. Therapeutic use refers to the treatment of established infections. Prophylaxis is the use of drugs in either individual or groups to prevent the development of infections. Therapeutic and prophylactic uses involve administration of drugs by different routes at therapeutic levels for short periods of time (Hirsh and Zee,1999). Growth promoter use is the use of antimicrobials/hormones as feed supplements to promote growth of food animals and enhance feed efficiency (Kanneene and Miller, 1997).

2.2.1. Prophylactic and therapeutic uses

Depending on action, prophylactic and therapeutic drugs may be divided in to different groups. The most widely distributed drugs are antimicrobials, antimycotic and antiparasitic preparations and their use in prevention and treatment of animal diseases is extremely important part of efficient production of food of animal origin. Veterinary drug products approved or licensed for use throughout the world do not vary greatly from country to country, although the levels of use, withdrawal times accepted safe level in food do change from country to country in almost all cases. Depending on the requirements in different countries, feed stuffs containing veterinary drugs may be available only on the prescription of a veterinarian or they may be freely available. In most circumstances, if recommended withdrawal periods are observed, this is not expected to lead to the presence of unacceptable residues (FAO, 1984).

2.2.2. Growth promoters

It is common practice in many countries to add antimicrobial drug products to animal feed for the purpose of increasing rates of weight gain and improving feed conversion efficiency. In some countries drugs not used for the therapeutic purposes are licensed for growth promotion use. In other countries, drugs used for therapy are also licensed for growth promotion (FAO, 1984).

It is estimated that about half of beef animals in the UK and more than 90% of feedlot cattle in USA are treated with growth promoters. On the other hand, many countries prohibit all uses of growth promoters and the use of estrogenic stilbenses such as diethylstilbestrol (stibestrol or des) and hexoestol are banned in most countries, however, in some countries the use of these compounds as growth promoters with hormonal activity is approved. Implants (at specific sites normally not used for human food) are allowed because they are believed to yield safer meat for the consumer than other preparations (FAO, 1984).

Antibiotic growth promoters are used to help growing animals to digest their food more efficiently, get maximum benefit from it and allow them to develop into strong and healthy individuals. Although the mechanism underlining their action is unclear, it is believed that the antibiotic suppress sensitive populations of bacteria in the intestines. It has been estimated that as much as 6% of the net energy in the diet could be lost due to microbial fermentation in the intestines (Jensen, 1998). It is also hypothesized that cytokines released during the immune response as a result of bacterial infection may stimulate the response of catabolic hormones, which could reduce muscle mass. Therefore, a reduction in gastro-intestinal infections would result in subsequent increase in muscle weight. Whatever the mechanism of action, the result of the use of growth promoters is resulting in meat of better quality, with less fat and increased protein content (Peter and John, 2001).

2.2.3. Other uses of veterinary drugs

Control of reproduction

Prostaglandins and their analogs and sex steroids are used to regulate fertility and breeding programs. Glucocortcoids and prostaglandins are used as abortifacients or to control timing of parturition. Animals would not be slaughtered shortly after the treatment and residue problem in meat could only occur in the event of casaulity meat slaughter (FAO, 1984).

Pre slaughter control of stress

Certain neuroleptic drugs (tranquilizers) are administered to avoid the excitement of animal or to curb aggressive behavior. The misuse of such drugs and some beta –adrenogenic blocking agents to reduce the stress of transportation to the slaughterhouse raises concern from the view point of consumer protection. Residues of drugs given for this purpose will remain at a high level in edible tissues, since animals are slaughtered shortly after the drug is administered and while the concentration of the drug remains at therapeutically effective levels (FAO, 1984).

Preservation and processing of food

In preservation and processing, food additives are added (employed) to prevent the onset of spoilage, to promote binding properties and to enhance flavor and nutritive value. These additives include antioxidants, sequestrants, coloring agents, stabilizers, sweeteners, tenderizers etc. At both production and processing stages, residues or contaminants may enter the food chain from intentional exposure to these chemicals (Gracey *et al*., 1999).

2.3. Significance of veterinary drug residues

2.3.1. Public health significance

No significant reported episodes of adverse human health effects occurring as a result of antimicrobial residues in foods when the veterinary drugs were used at the correct dosages and at the levels permitted. However, this does not necessarily mean that no adverse health effects have occurred. Drugs are intended to be toxic to various forms of microorganisms as such may have inherent toxic, mutagenic, teratogenic or carcinogenic effects to humans (FAO/OIE/WHO, 2003).

2.3.2. Development of drug resistance (microbiological properties)

Human health can either be affected through residues of drugs in food of animal origin, which may cause direct side effects, or indirectly, through selection of antibiotic resistance determinant that may spread human pathogen. In general, the effect of antibiotic residues in food of animal origin is significant when compared with the misuse of selection and amplification of antibiotic resistant strains of bacteria (Peter and John, 2001). Resistant microorganisms can get access to human either through direct contact or indirectly via meat, milk and/or egg. As the bacteria of endogenous flora of food animal contaminate food of animal origin, they might either colonize human or transfer resistance genes to humans endogenous flora and superimpose an additional load to the reservoirs of resistance genes already present in man (Stobberingh and Bogaard, 2000)

The use of antibiotics in food animals can result in antibiotic resistance bacteria reaching the human population through variety of routes. Antimicrobial resistant bacteria such as *E. coli* can colonize intestines of people. Heavily exposed humans (farmers, who use food containing antibiotics, slaughterhouse workers, cookers and other food handlers) often have a higher incidence of resistant *E .coli* in their feces than the general population. Contaminated meat by intestinal bacteria at slighter is extensive and an important route by which resistant bacteria reach people. While many bacteria are nonpathogenic, some pathogenic bacterial species from the intestines of animals cause zoonotic infection to human such as *Salmonella* species*, Campylobacter jejuni* and these infection may be harder to treat because of acquired resistance by

humans are a potential sources of resistance plasmids for human pathogenic bacteria other than the zoonotic infection (Hirsh and Zee, 1999).

A close relationship between tetracycline streptomycin, gentamycine and chloramphenicol residues and the resistance of bacteria isolated from the samples was found, suggesting that the presence of low level of antimicrobials might exert a positive pressure towards the selection and expression of resistance in bacteria colonizing animal tissues (Vazquez-Moreno *et al*., 1990). It is possible, although not proven, that low doses of antimicrobial drugs could alter intestinal enzyme activity and have an effect on certain hormones and drugs (Gorbach, 1993).

2.3.3. Immunopathological aspects (hypersensitivity reactions)

Drug hypersensitivity is defined as an immune-mediated response to a drug agent in a sensitized patient, and drug allergy is restricted to a reaction mediated by IgE (Riedl and Casillas, 2003). Drugs are foreign molecules, but their molecular weight is usually too small to be immunogenic, they must act as haptens, which must combine with carrier proteins to be immunogenic and elicit antibody formation (Dewdney *et al*., 1991). Immunogenic reactions may manifest from life-threatening anaphylactic reactions to milder reactions, such as rashes. Drug induced allergic reactions may occur acutely (within 60 minutes of challenge), subacutely (1-24 hours), or as latent responses (1 day to several weeks). The acute and some subacute disorders are often due to type I (IgE)-mediated reactions and more rarely, due to IgG antibodies (type II). Immune complex disorders (type III) are much rarer in this context. Type IV (cell mediated) responses develop more slowly. The principal types of disorder are: type I: anaphylactic shock, asthma and angioneurotic edema: type II: hemolytic anemia and agranulocytosis; type III: serum sickness and allergic vasculitis, and type IV: allergic dermatitis (Dayan, 1993; Riedl and Casillas, 2003).

In anaphylaxis exposure rapidly leads to severe acute bronchoconstriction, often risking a degree of asphyxia, marked hypotension, possibly edema at the site challenges, and severe general illness (Dayan, 1993). An allergic reaction may be triggered by antimicrobial residues in a previously sensitized individual. In relation to primary sensitization, it is unlikely that residues could be attributed to the overall immune response in view of the very low concentrations that are likely to be encountered. The duration of exposure is also short (Dewdney *et al*.,1991; Sundlof *et*

al., 2000). Notwithstanding their non toxic nature, β-lactams appear to be responsible for most of the reported human allergic reactions to antimicrobials (WHO, 1991; Sundlof, 1994; Fein *et al.*, 1995). Aminoglycosides, sulphonamides and tetracyclines may also cause allergic reactions (Paige *et al*., 1997). Certain macrolides may also in exceptional cases be responsible for liver injuries, caused by a specific allergic response to macrolide metabolite modified hepatic cells (Dewdney *et al.,* 1991).

2.3.4. Carcinogenic effects

Carcinogenic effects refer to an effect produced by a drug having carcinogenic or cancer producing activity. Among the carcinogenic veterinary drugs in current use in many countries are nitrofurans, nitroimidazols quinoxaline-n-dioxide, grisofulvin, and some stilbene derivatives. These drugs are acquired via food of animal origin as antimicrobial residues. The potential hazard of carcinogenic residues is related to their interaction or covalent binding with various intracellular compounds such as proteins, ribonucleic acid, glycogen, phospholipids and glutathione. Covalent binding or transformation of a chemical receptor complex (adduct) occurs; dissociation of the adduct back to the free chemical and the free receptor does not occur. Consequently, covalent adducts are irreversible. This lead to damage of cellular components such as DNA. A very important generalization that appears to be surfacing from the chemical carcinogenesis literature is that virtually all chemical carcinogens that initiate neoplastic lesions are either electrophilic or capable of being metabolized to products that are highly electrophilic (Booth and McDonald, 1988).

2.3.5. Mutagenic effects

The term mutagen is used to describe chemical agents that damage the genetic components of a cell or an organism. Genetic material of all living organisms, with exception of some virus is DNA. Several chemicals including alkylating agents and analogs of DNA bases have been shown to elicit mutagenic activities. There has been an increasing concern that drugs as well as environment chemicals may pose a potential hazard to the human population by production of gene mutations or chromosome abrasions. Three types of genetic injury have been recognized: point mutation, gene elimination, and chromosome breakage. Either the germinal or somatic may

be affected. Understandably, injury to either cell group may lead to a serious of consequences. However, from a public point, mutation in the germinal cells are of more immediate importance because of the hazard to the further generation. Many mutagens also have carcinogenic activity example, the polycyclic hydrocarbons that are man made chemicals and naturally occurring products such as, aflatoxins and related compounds, have mutagenic and carcinogenic properties. Humans can not totally escape exposure to mutagenic agents even if dietary and environmental sources could be free of them. A mutagen, (S)-3-(1,3,5,7-dodecaoentaeny(oxy)-1,2-propanediol, is produced by at least five species of intestinal bacteria. The compound is associated with colon cancer; diet high in fiber are thought to disease risk (Booth and McDonald, 1988).

2.3.6. Teratogenic effects

The term teratogen applies to a drug or chemical agent that produces a toxic effect on the embryo or fetus during a critical period of gestation. As a consequence, a congenital malformation that affects structural and functional integrity of the organism is produced. The well known thalidomide incident involving a number of children in Europe was a direct testimony to the hazards that may occur when such an agent is administered during pregnancy. Approximately five years after the introduction of thalidomide into clinical use, this drug was identified as etiological agent phacomelia “Seal limbs” (Booth and McDonald, 1988).

2.3.7. Other harmful effects

Hazards of chloramphenicol observed in association with clinical use in humans include dose-related, reversible suppression of the bone marrow, gray baby syndrome, which is a circulatory collapse in children less than 30 days on high doses, and irreversible, distinctive, non-dose related aplastic anemia (WHO, 1988; Waltner-Toews and McEwen, 1994). Aplastic anemia can occur in susceptible individuals exposed to concentrations of chloramphenicol that might remain as residues in edible tissues of chloramphenicol-treated animals (Settepani, 1984). Aminoglycosides can produce damage in urinary, vestibular and auditory functions (Aerts *et al*., 1995).

2.4. Economic impacts of drug residue

Antimicrobial residues in food of animal origin can have a determinant effect on the processing of cultured products such as cheese thus affecting a dairy industry (Brady,1982). A wide spread availability and use of antimicrobials have several negative implications on global health care; among these drug resistance is the most important one. The primary economic implication of resistance on the diminishing efficacy of antibiotic treatment includes the need to rely on more expensive drugs that may be practically unaffordable for most primary health care problems (WHO, 2001).

Drug residue remains very significant from the prospective of international trade and consumer confidence, because it results in international trade barrier. As tariffs are removed and goods flow freely between countries, importing countries must be in confident that goods available for purchase are safe, and in addition to this, from time to time, there is pressure to use antimicrobial residues on notariff barrier to import (Kanneene and Miller, 1997). Major economic losses and animal welfare problems could also arise in veterinary medicine because antimicrobial resistance has been found to cause therapy failure and higher mortality rate (Holmberg *et al*., 1987).

2.5. Safety evaluation

To asses the safety of ingested antimicrobial residues national and international committee evaluated data on chemical, pharmacological, toxicological and other properties; e.g. antimicrobial, properties of drugs derived from studies of experimental animals and observations in humans (Woodward, 1998). Various organizations and regulatory authorities have developed methods for, and adopted regulatory approaches to, evaluating the safety of edible foodstuffs derived from animals treated with specific drug, while regulatory approaches vary among international authorities and agencies, their objective encompass evaluation decisions (FAO/OIE/WHO, 2003).

2.5.1. Acceptable daily intake (ADI)

The term acceptable daily intake is defined as an estimate of the residue, expressed on a body weight basis, that can be ingested daily over a life time without any appreciable health risk (EC, 2001). Calculation of ADI is based on array of toxicological safety evaluation that take into account acute and long term exposure to the drug and its potential impact, such as carcinogenecity, genotoxicity, reproductive toxicity, teratology, neurotoxicity, immunotoxicity and allergenecity, ocular toxicity, cardiac toxicity and in case of antimicrobial agents, safety for gastrointestinal microflora. These resulting in a defined maximum quantity which may be consumed daily by even the most sensitive group in the population without any outward effects the ADI is determined as a consecutive estimate of safe ingestion levels by the human population based on the lowest "no effect level" (NOEL) among battery of toxicological safety studies (FAO/WHO/OIE, 2003). The ADI was calculated by dividing this (NOEL) by a suitable safety factor, usually 100 which assumes that humans are 10 times more sensitive than animals and that within the human population there is a 10 fold range sensitivity (IPCS, 1987; Woodward, 1998).

2.5.2. Maximum residue limits (MRLs)

The maximum residue limit is the maximum concentration of residue resulting from the use of a veterinary medicinal product which may be legally permitted or recognized as acceptable in or on a food, allocated to individual food commodities. It is generally accepted that the MRL of analyte of any foodstuff (Gracey *et al.,* 1999) is determined by:

1. A minimum dose which produces detectable effects in experimental or which, in a therapeutic preparation used in human medicine, produce a recognizable effects.
2. A safety factor in the range 10:1000 and which is lower (1:10) if a preparation is already acceptable in human medicine or higher (1:1000) if there is any evidence to indicate a special risk from experience with chemically similar compounds
3. A series of factors to balance the proportion of the particular tissues in the average diet.

Because of the impossibility to find a threshold concentration for chloramphenicol in induction of aplastic anemia in humans, due among other things to a lack of results from residues and toxicity

test studies, no ADI could be set. Substances for which no maximum residue level can be established because residues of these substances, at whatever limit, in foodstuffs of animal origin constitute a hazard to health of the consumer (EEC, 1990). Maximum residue level of some veterinary drugs are shown in Table 1 and 2.

Table 1. Antibiotic maximum residue limits (MRL) in bovine tissues (concentration (μg/kg)

Compound	**Target tissue**				
	muscle,	liver	kidney	fat	milk
Sulphonamides	100	100	100	100	*
Benzyl penicillin	50	50	50	50	4
Ampicillin	50	50	50	50	4
Apramycin	1000	10000	20000	1000	*
Cefquinone	50	100	200	50	*
Cloxacillin	300	300	30	30	30
Erythromycin	400	400	400	400	40
Florenicol	200	3000	300		*
Spiramycin	200	300	300	300	200
Streptomycin	500	500	1000	500	200
Tetracycline	100	300	600		100
Trimethoprim	50	50	50	50	50
Tylosin	100	100	100	100	50

Source: Gracey *et al*., 1999; * Not determined

Table 2. Maximum residue limits of common anthelmintics in tissues of different livestock species (μg/kg)

Anthelmintics	Species	Target tissue	MRLs
Levamisole	Bovine, ovine, porcine poultry	Muscle, kidney, fat	10
		Fat	100
Ivermectin	Ovine	Liver	15
	Porcine	Fat	20
	Bovine	Liver	100
Abamectin	Bovine	Liver	20
		Fat	10
Doramectin	Bovine	Liver	15
		Fat	25
Closantel	Bovine	Muscle liver	1000
		Kidney, fat	3000
	Ovine	Muscle liver	1500
		Kidney	5000
Febantel, fenbendazole	All-food producing animals	Fat	2000
		Liver	1000
		Muscle, kidney, fat	10
Oxfendazole	Bovine, ovine	Milk	10
Triclabendazole	Bovine, ovine	Muscle, kidney, liver	150
		Fat	50
Thiabedazole	Bovine, ovine, caprine	Muscle	100

Source: Gracey *et al*.(1999)

2.5.3. Withdrawal period

To ensure that drug residues have declined to a safe concentration following the use of drugs in animals, a specified period of drug-withdrawal must be observed prior to providing any products for human consumption. Withdrawal period is the time which passes between the last dose given

to the animal and the time when the concentration of residues in the tissue: muscle, liver, kidney, skin, fat, milk, eggs, and honey is lower than or equal to the MRL (Jackson, 1980).

Table 3. Withdrawal times and discard times of milk for injectable drugs used

Drugs	**Pre-slaughter withdrawal time(days)**	**Discard times for milk (hr)**
Amoxicillin rihydrate	25	*
Ampicillin trihydrate	6	48
Erythromycin	14	72
Furosamide	2	48
Hydrochlorothiazine	*	72
Ivermetin	35	*
Levamisole phosphate	7	*
Oxytetracyclinehydrochloride	28	*
Procaine penicillinG	10	*
Benzanthine penicillin G	30	*
Dihdrosterptomycine sulphate	30	72
Sulphadimethoxine	5	60
Sulphaethoxypyridazine	16	72

Source: Booth and McDonald (1988); * Not determined

2.6. Tetracyclines in the public health and veterinary practices

The tetracyclines, among the first of the antibiotics to become available 50 years ago, are still widely used. It is a kind of antibiotics which is produced by the bacteria of the genus *Streptomyces.* They are effective against a wide range of gram- positive and gram- negative bacteria, interfering with protein synthesis in these microorganisms. Tetracycline is used to treat many bacterial infections ,such as rocky Mountain, spotted fever, some eyes, respiratory, intestinal, bovine mastitis urinary infections, etc and are added at sub therapeutic levels to cattle feeds for prophylaxis and, some kinds of acne, and some diseases especially the infecting

microorganisms is resistant to penicillin. The first drug of the tetracycline family, chlortetracycline, was introduced in 1947 (Wikipedia Encyclopedia,2004). The term tetracycline obviously implies its chemical structure with four (tetra-)cycles as follows:

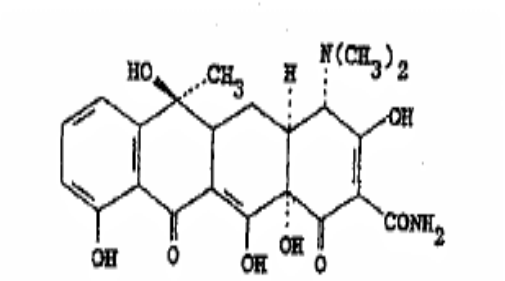


Figure 1. Structural formula of tetracycline
Source: Wikipedia Encyclopedia (2004)

The use of antibiotics may result in drug residues in the meat, especially if they are not used according to label directions. The presence of antibiotic residues in meat, milk, etc. may cause allergic reactions in sensitive individuals. However, the use of this compound may result in residues in animal derived food products, especially if proper withdrawal times for treated animals have not been used (Long *etal*.,1990). These residues may pose a health threat to consumers, depending on the type of food and the amount of residue present. For this reason, regulatory agencies have established maximum legal tolerance levels for tetracycline drug in animal derived food products (USDA,1988).Tetracycline residues that exceed the tolerance level is a potential hazard to human health. Some individuals may have an allergic reaction to these compounds, and/or resistance by some bacteria may be induced. Treatment with tetracyclines during the second month of pregnancy presents a teratogenic risk to the fetus (Czeizel *eta*l.,1998) As undesirable side-effects, tetracyclines not only discolor the primary and permanent teeth but also causes hypoplasia in developing teeth when administered to infants, mothers during the last two trimesters of pregnancy and children under 12 years of age. It has also been suggested that discoloration caused by tetracyclines occurs in adult dentition (Tanase *et al.,*1998). Children receiving tetracyclines may develop brown discoloration of the teeth. The risk of this untoward effect is highest when tetracyclines are given to neonates and babies prior to the first dentition. However, pigmentation of the permanent dentition may develop if the drug is given between the

ages of 2 months and 12 years, when these teeth are being calcified. An early characteristic of this defect is a yellow fluorescence of the dental pigment, which has an ultraviolet spectrum with an absorption peak at 270 nm. The deposition of the drug in the teeth and bones is probably due to its chelating property and the formation of a tetracycline-calcium orthophosphate complex. As time progresses, the yellow fluorescence are replaced by a no fluorescent brown color that may represent an oxidation product of the antibiotic, the formation of which is hastened by light. Treatment of pregnant women with OTC may also produce discoloration of the teeth in their children (Theodore *et al*.,1990).

The 36th Joint FAO/WHO Expert Committee on Food Additives (JECFA) meeting in 1990 established MRL for oxytetracycline of 600 µg/kg in kidney; 300 µg/kg in liver; 100 µg/kg in muscle; 100 µg/kg in milk; 200 µg/kg in eggs; and 10 µg/kg in fat for all species for which residue depletion data were provided (cattle, swine, sheep, chickens, turkeys and fish). These MRLs were approved through the Codex Alimetarius Commission in 1994.The residue distribution for tetracycline, chlortetracycline and oxytetracycline in food producing animals is comparable (EMEA,1995). The ADI of 0-3 µg/kg of body weight previously allocated to oxytetracycline was converted to a group ADI with chlortetracycline and tetracycline at that meeting. It was recommended that the MRL of 10 µg/kg for oxytetracycline in fat be withdrawn and that MRLs in fat for chlortetracycline and tetracycline are not required. This recommendation was raised based on the evidence that tetracyclines have the affinity to the liver, spleen, bone marrow, teeth; but diffusion in liquor and in fatty tissues is poor. That is why an MRLs for tetracyclines in fat is not really necessary (Forth *et al*.,1983). Allocated MRLs for tetracyclines can be satisfactorily monitored by a combination of the microbiological and chemical (identification and quantification) analyses that are potentially available. Target tissues for the analysis of all tetracyclines are kidney and muscle in cattle, pigs and poultry and based on limited data, kidney is the target tissue in sheep (FAO.1997).

In addition, tetracyclines are poorly metabolized in animals (Nielsen and Hansen,1996).therefore, they can also occur in animal slurry that may pollute the environment (Sczesny *et al*.,2003). However, a potential risk for the environment cannot be assed yet as very little is known about the not excludible casual concentrations of antibiotics (Hirsch *et al.,*1999).

2.7. Detection of residues in food

Residue testing can occur either before or after slaughter. Whenever possible, testing animals before slaughter is desirable, since this provide the opportunity to hold the animal until the withdrawal time is met (OIE, 1997). To provide greatest flexibility in the laboratory analyses in National Surveillance Scheme testing, a complete set of samples contain the following: 50ml of blood, 250gm of kidney, 50gm of diaphragmatic muscle, 50ml of bile and 50ml of urine. After collection, samples should be cooled rapidly, placed in an insulated container containing frozen freezella pack and dispatch to the laboratory. The samples are recorded and then placed in a – 20 ^{0}C freezer for storage before testing (Gracey *et al.,* 1999).

2.7.1. Screening methods

These methods are the first hand analysis of the sample to establish the presence or absence of residues (Aerts *et al.*, 1995). They are aimed at avoiding false negative results while false positive results are tolerable. These tests when used for substances with an established maximum residue limit, it must be sufficiently low to detect residues at this level but for substances which are not authorized for use in food producing animals the limit of detection should be as low as possible. Besides, screening tests should be oriented to have high sample throughput capability and low cost because of their convenience and broad spectrum characteristics (Aerts *et al.*, 1995; Haasnoot *et al.*, 1999). Microbiological tests are not specific, indicating only the presence of an inhibiting agent. If organ or tissue, renal pelvis fluid, urine etc is used as test matrix, the result is expected to predict residue concentrations in muscle tissue. The reason for the use of other test matrices than tissue is that higher residue concentrations are usually found in those tissues (Aerts *et al.,* 1995).

Agar diffusion tests

In agar diffusion tests the agar is inoculated in standardized manner, and the sample is applied to the agar surfaces producing clear inhibition zone within short period of time as shown in Figure 1. During the first hours of diffusion, the concentration of antimicrobial within agar medium at the edge of the sample is relatively high and diminishes sharply at increasing distance from the sample. The slope of the concentration gradient levels off, resulting in a broader gradient of

decreasing concentration within the agar medium. The result of diffusion-inhibition test is the result of a race between spreading of antimicrobial by diffusion and grow-out of the test organisms. When using spores the moment at which the germinating spores reach some critical stages perhaps-where the resulting vegetative organisms first divides is the "timing" mechanism for determining the position of the zone edge. Within the zone the germinating organisms are prevented from passing the critical stage, where as outside the zone the concentration of antimicrobial is too low to prevent the event. The rate of diffusion through an agar gel depends up on the concentration of drug in the sample, the size and shape of antimicrobial molecule, the viscosity of the agar gel, pre-incubation time and temperature. Inhibition zone width can be measured with rulers or computerized image analysis system (Schoevers *et al*., 1994).

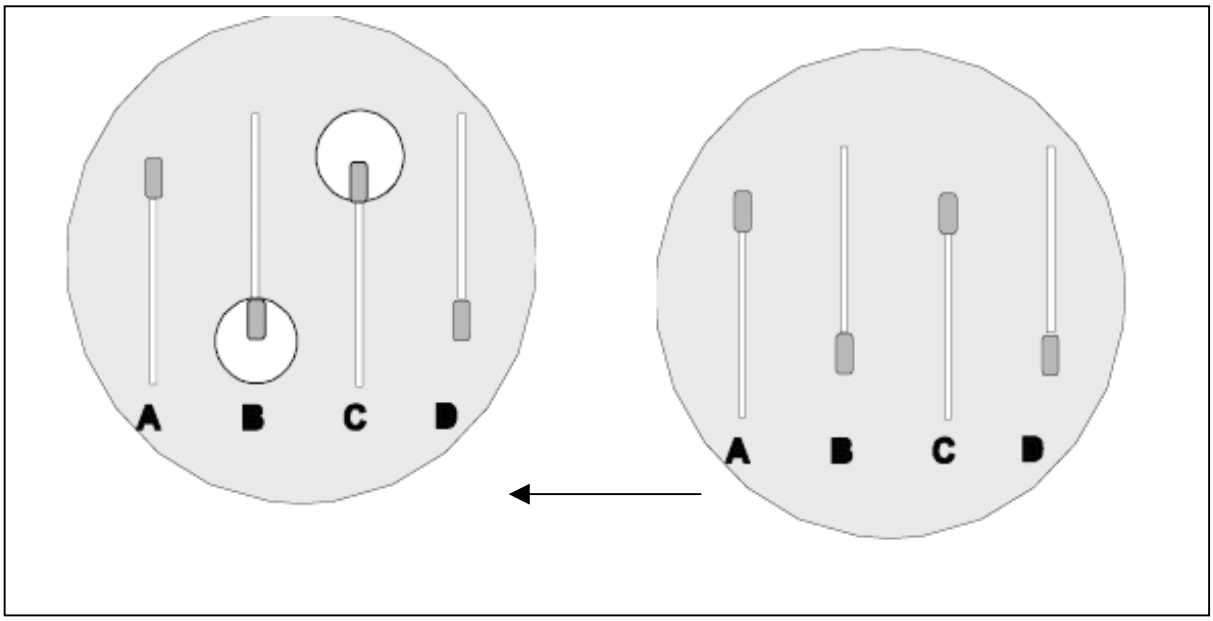


Figure 2. Agar diffusion test due to inhibitory effect of antibiotic in a sample.

Source: Dey *et al*.,1998

In agar diffusion tests, the agar medium influences the zone size by its effect on the activity and the rate of diffusion of the antimicrobial, and its effect on the growth rate of the test organism. The general capacity of the medium will influence the length of the lag phase and the generation time of the test organism; nutritionally deficient media produce larger inhibition zones because of a prolonged lag phase. The pH of the medium affects the activity of certain antimicrobials. The mechanism of the effect of pH on antimicrobial activity are incompletely understood and

inconsistent from drug to drug (Amsterdam,1996). Each of the current and potential screening tests has limitations i.e. additional screening test are required for these substances, they can not be used to detect banned antimicrobial present in low concentration, for example, chloramphenicol and sulphonamides have to be in relatively high concentration for detection (>MRL) (Korsrud *et al*., 1998).

Table 4. Some currently used antimicrobial residue screening tests

Test method	Test bacterium	Test medium	Medium pH	Additional substances	Test matrix	Reference
Four plate test (FPT)	*B .subtilis* BGA, *M. luteus* ATTC 941	Test agar	6.0,7.2, 8.0	Trimethoprim (pH 7.2)	Muscle, kidney liver	Bogaerts and Wolf (1980)
Swab Test on Premises (STOP)	*B. subtilis*	Antibiotic medium No 5	7.9		Muscle, kidney Liver tissue fluid	Johnston *et al* (1981)
Two-plate test	*B. subtilis*	Test agar	6.0, 8.0	Trimethoprim (pH 8.0)	Muscle, kidney	MAFF (2001)
The Live Animal Swab Test (LAST)	*B. subtilis* ATTC 6633	Antibiotic medium No 5	7.9		Urine from live animal	USDA (1983)
New Dutch Kidney Test (NDKT)	*B. subtilis* BGA	Standard nutrient agar	7.0	Dextrose, Phosphate buffers, Trimethoprim	Renal pelvis fluid	Nouws (1988)
Belgian Kidney Test (BKT)	*B. subtilis* BGA	Standard nutrient agar	7.0	Dextrose, Trimethoprim	Muscle, kidney	Koenen-Dierick *et al* (1995)
Calf Antibiotic and Sulfa Test (CAST)	*B .megaterium*	Mueller-Hinton agar	7.4	Dextrose, bromocesol purple	Muscle, kidney Liver tissue fluid	USDA (1984)
The Fast Antibiotic Screen Test (FAST)	*B. megaterium*	Mueller-Hinton agar	7.4		kidney tissue fluid	USDA (1994)
Premi®Test	*B. stearothermohilus*				Muscle, kidney liver, urine	USDA (1994)

Immuno assay screening tests

Immunological methods are based on the ability of antibodies to bind specifically to different substances. The binding forces are weak molecular interactions like coulmb and Vander Waals forces as well as hydrogen bonding and hydrophobic binding. Veterinary drugs, being low molecular weight compounds, generally do not prompt any immunological response in animals. To produce such a response, the compounds have to be coupled to a large molecule such as bovine serum albumin (Haagsma and VanDeWater, 1992). On the basis of antigen-antibody reaction, enzyme linked immunological screen tests are available to detect chloramphenicol, gentamycin, neomycin, tylosin, sulphamethazone, tetracycline in meat and poultry. On site immuno assay for detection of drug residue will mainly be solid phase indirect competitive enzyme immuno assays. Antibody to a drug is immobilized on a solid support such as filter membranes. Sample is added, and if no drug is in the sample, no binding of the antibody will take place. Drug coupled to an enzyme such as horse radish peroxidase is then added to the filter membrane. Because no drug was in the sample, the enzyme labeled drug is able to bind to the antibody and remain at the reaction site. In the last step an enzyme substrate such as urea peroxidase and 4-CAF (chloramphenicol) is added, and the bound enzyme hydrolyses the substrate to produce a colored precipitation at the reaction site. The test result is positive if the sample spot remains colorless, and negative if it turns to blue color (Gerald and Joseph, 1991).

The other test under this group is radioimmno assay (the Charm II test) which uses bacteria with specific receptor sites for antimicrobials or an antibody coating, and a radio active labeled antimicrobial. The bacteria are added to sample and binding reaction occurs between drug functional groups and receptor sites on microbial cells. A radio active labeled analyte competes with incurred drug residue for receptor sites. A liquid scintillation counter is used to measure bound ^{14}C or ^{3}H from the labeled drug. Labeled drug not bound to receptor sites is removed from the substrate prior to counting, and therefore the greater the amount of incurred drug present in the sample, the lower the count and compared to the previously determined control point or to standard curve. The test can be used both for the detection and determination of antimicrobial residues (Nouws *et al*., 1998).

2.7.2. Chromatographic analysis

Chromatography is the collective term for a family of laboratory techniques for the separation of mixtures. It involves passing a mixture dissolved in a mobile phase (which moves in a definite direction) through a stationary phase (immobilized on the support particles), which separates the analyte to be measured from other molecules in the mixture and allows it to be isolated (Wikipedia Encyclopedia, 2004).These methods provide full or complementary information enabling the analyte to be identified unequivocally at the level of interest. The tests are employed to determine presence or absence of residues in a sample found positive by routine screening test. These test are aimed at preventing false positive results as well as having an acceptable low probability of false negative results (Heitzman, 1994).

Commonly used procedures for the detection of veterinary drug residues include HPLC, gas chromatography (GC), thin layer chromatography (TLC) and mass spectrometry (MS). Chemical methods usually proceed with a preliminary extraction in order to isolate drug of interest from biological matrix. The main objectives of sample treatment are removal of macromolecules and other matrix constituents that may either adversely affect the chromatographic systems or interfere with the detection and enrichment of the analytes in order to achieve the required low limits of detection. Compounds must be separated from another and food matrices (Aerts *et al*., 1995). The low solubility of some antimicrobials in organic solvents has made it difficult to develop procedures to extract and concentrate their residues from biological matrices. Other antimicrobial are either insufficiently volatile or too thermally unstable (or both) to permit their analysis using GC or MS. Liquid chromatography has merged as the method of choice for determination of antimicrobial which are rather polar, non volatile, and some times heat stable (Shaikh and Moats, 1993).

Approaches to extraction include extraction with water or buffer, direct solid phase extraction, deproteinization with tungstic acid, trichloroacetic acid, water-miscible organic solvents such as methanol, acetone or acetonitrile combined with buffers or acids, ultra filtration, partitioning into water-immiscible organic solvents, heating to denature proteins, and extraction with superficial carbon dioxide. With the automated sequential trace enrichment of dialysates sample

pretreatment is restricted to homogenization and dilution of the samples; clean-up is by on line dialysis and on-line solid phase extraction. Sample clean-up procedures includes column chromatography, (thin layer liquid chromatography) TLC, liquid-liquid extraction, solid phase extraction and solid phase dispersion(Moats, 1997).

The aim of chromatography in general is the resolution or separation of different molecular species (Klassen and Edberg, 1996). The mobile phase passes over the stationary phase at a constant rate; the two phases possess different chemical properties. As the analytes in the mobile phase pass over the stationary phase, those with polarity closer to that of the stationary phase are retained selectively for a time on the column. Conversely, the analyte molecules with polarity closer to that of the mobile phase tend to remain in the mobile phase, passing through the column faster. Passing through the instrument monitor sequentially, these "groups" of molecules give rise to peaks on the chromatogram (Klassen and Edberg, 1996). HPLC methods utilize the same basic steps: extraction of the drug with a specific solvent, separation of the drug on the solid phase, detection of the effluent from the solid phase by spectrometry and quantitation of the amount of antimicrobial present by peak height or peak area analysis. Of the detection methods used with LC analysis, UV absorbance is the simplest and most widely used. Derivatization, either pre- or post column, is frequently used to enhance UV absorbance or to form fluorescent compounds. Compounds with little or no UV absorbance require other approaches (Moats, 1997). Lanthanide sensitized luminescence is an alternative to UV detection and other luminescence techniques, i.e., fluorescence and phosphorescence, in separation science for the detection of drugs (Rietourd et al., 1997; Hernández-Arteseros *et a*l., 2000).

TLC methods can be used to separate mixtures of inorganic ions, organic molecules and bioorganic compounds such as pigments, lipids, amino acids, nucleotides and sugars. The TLC plate typically consists of a different millimeter thick layers of adsorbent material bonded to a glass, aluminum or plastic support. These surfaces provide a large area for chromatographic separation. After a small volume of sample solution is applied to the adsorbent surface and allowed to dry, the plate is placed in a beaker or tank containing the appropriate solvent. Only the edge of the plate nearest the samples is in contact with the solvent. The solvent is drawn into the dry adsorbent material and travels up the plate through the samples. The migration rate of the

sample components over the adsorbent depends on their chemical structure. TLC involves a stationary phase of a thin layer of adsorbent like silica gel, alumina, or cellulose on a flat, inert substrate. Most adsorption materials can have surface charges. Thin layer chromatography is a technique that involves placing a small dot of sample solution onto TLC plate. The plate is placed in a chamber containing a shallow layer of solvent and sealed. As the solvent rises through the plate it meets the sample mixture which starts to travel up the plate with the solvent. Different compounds in the sample mixture travel different distances according to how strongly they interact with the stationary phase. This allows the calculation of retention factor (R_f) (a measure of the speed at which a substance moves in a chromatographic system) value and can be compared to standard compounds to aid in the identification of an unknown substance (Annex 3).Substances that are adsorbed to these materials are polar or polarizable molecules. An example of a polar molecule is water. The oxygen atom has slightly more negative charge than the hydrogen atoms, which consequently have slightly more positive charge. This is because the oxygen nucleus attracts the negatively charged electrons in the chemical bonds more strongly than the hydrogen nuclei. Therefore, even though the water molecule is overall electrically neutral, its individual atoms do possess partial negative or positive charge. Molecules that exhibit these properties are called polar. Molecules that contain opposite charges, or partial opposite charges, possess a dipole. The adsorbent material has many polar and fully charged (ionic) chemical groups on its surface. Polar sample molecules can interact with these groups by dipole-dipole and dipole-ion interactions. These interactions involve the attraction between regions with opposite charges. Many types of molecules can have a net positive or negative charge. Charged sample molecules interact with the adsorption surface by the same chemical forces discussed. TLC plates can also be prepared to contain a large amount of chemical groups with net positive or negative charges on the surface. Charged sample molecules can be efficiently separated on these plates with solvents having the appropriate pH and salt concentrations. This type of TLC is called ion exchange and involves the interaction of compounds with opposite net charges. Ion-exchange TLC typically involves stronger interactions than adsorption TLC. TLC is generally very sensitive to small differences in chemical structure. The structure affects the strength and type of interactions between sample and adsorbent (Sherma and Fried,1996).

2.8. Prevention of veterinary drug residues

Dairy and beef producers must be able to produce milk and meat that continuously stand the test of critical consumer and government security. It is therefore, effectual for veterinarians to follow quality control procedures to prevent entry of violative residues in meat or milk intended for human consumption. Proper use guide developed for use by dairy and beef producers include the following (Radostits, 1994; AVMA and NMPF, 1991; IDF,1995):

1. Herd health management: All food animals should be maintained in a clean healthy environment whenever possible. A nutritional program should in effect to meet growth, maintenance, and lactation needs. The veterinarian should implement a health program that encompasses preventive medical procedures. Drug residues are best avoided by implementing management practices and health programs that keeps animals healthy and producing efficiently.
2. Use of approved drugs: Dairy and beef producers should not use or store unapproved drugs, special mixes, or products with inadequate labels as unapproved drugs have no data regarding efficiency, safety or withholding times. The herd veterinarian should be certain that extra-label drug use involve only approved products.
3. Establish a valid veterinary /client/ patient relationship: The use of prescription drugs and extra-label use of drugs necessitates a veterinarian /client /patient relationship, which is established; hence a veterinarian works closely with owner in health management of the herd.

4. Restriction of feeding antibiotics to extreme ages: Antibiotics are effective only during early growth phase of the animal. Addition of antibiotic to feed should generally be restricted to age spans indicated by WHO (WHO,1963). Feeding antibiotics beyond these age spans, which is represented in Table 5, not only increases the probability for accumulation of violative residues in tissues, but economically unsound. Now a days Europe is far ahead of the United States in the responsible use of antibiotics. On January 1, 2006, the European Union banned the feeding of all antibiotics and related drugs to livestock for growth promotion purposes. The sweeping new policy follows up a 1998 ban on the feeding of antibiotics that are valuable in human medicine to livestock for growth promotion. Now, no antibiotics can be used in European livestock for growth promotion purposes. It is part of the European Commission's overall

strategy to tackle the emergence of bacteria and other microbes resistant to antibiotics, due to their overexploitation or misuse (Union of Concerned Scientists, 2006)

Table 5. Recommendations for feeding restrictions of antibiotics to animals

Species	**Age**
Poultry	8-10 months
Swine	4-10 months
Beef cattle	18 months
Lambs	2 months

Source: Booth and McDonald (1988)

5. Proper drug administration and identification of treated animals: Before administration or dispensing a drug one has to consider the following:
   1. What drugs are approved for all classes of cattle on farm
   2. Be familiar with approved dosages
   3. Be familiar with route of administration
   4. Be familiar with withdrawal time

   Increasing dosage frequency of administration, changing the route of administration, or changing duration of treatment constitute extra label use which affect the withdrawal time, thereby increasing the chance of residues.
6. Proper maintenance of treatment records and identification of treated animals: Institute a workable health record for each animal to record all health related events, including administration. Record the identification of all treated animals in the permanent health record book.
7. Having Proper drug residue testing capabilities really available on and off the farm. This control point addresses the conditions under which residue testing should be considered; the proper selection and interpretations and potential misuse of residue tests.
8. Creating awareness of proper drug use and method to avoid marketing adulterated products.

3. MATERIALS AND METHODS

3.1. Study areas

The study was undertaken at Addis Ababa, Debre Zeit and Nazareth public slaughterhouses from October 2006 to May 2007.

3.1.1. Addis Ababa

Addis Ababa is the capital city and administration center for Federal Democratic Republic of Ethiopia. It lies in the central high lands of Ethiopia at an altitude of 2500 meters above the sea level and has an estimated human population of about 3 million. The average annual temperature and rain fall are 21 ^{o}C and 1800 mm respectively. Addis Ababa has a relative humidity varying between 70% to 80% during the rainy season and 40% to 50% during dry season (NMSA, 2003).

3.1.2. Debre Zeit

The town is located at 9ºN and 40ºE 47 km south east of Addis Ababa with a human population of about 95,000 people. The altitude is about 1850 m above sea level. It is an important small town where most governmental institutions, national and international research centers are located. The single major soil type of the area is vertisol, which covers about 60.8% of the district. Other soil types are cambisols and luvisols (23.5%) and rendzinas and phaeozems (14.7%). It experiences a bimodal pattern of rainfall with the main rainy season extends from June to September (of which 84% of rain is expected) and a short rainy season from March to May with an average rainfall of about 800 mm. The mean annual minimum and maximum temperatures are 12.3 ^{o}C and 27.7 ^{o}C respectively with an overall average of 18.7ºC (NMSA, 2003; CSA, 2001). Highest temperatures are reached in May and the mean relative humidity is 61.3%.

3.1.3. Nazareth

It is located at 39.17^0Nand 8.33^0 with an altitude of 1622 meters a.s.l. Nazareth is situated in a well known East African Rift valley system. The area has an annual rain fall and temperature ranges of 400-800mm and 13.9 to 27.7^oc, respectively (National Service Agency,1999/2000).The livestock population of the area is estimated at 7000, 36142 sheep and 42968 goats (Nazareth Agricultural Office,2003)

3.2. Study populations

The study population consisted of apparently healthy slaughtered cattle at Addis Ababa ,Debre Zeit and Nazareth public slaughterhouses. The cattle slaughtered at Addis Ababa slaughterhouse originated from different parts of the country; mainly from North Shewa of Amhara and Oromia region, Jimma, Gojjam, Wollo, East Showa, Wollega, West Showa, Afar, Somali, Borena and Arsi. The cattle for Nazareth slaughterhouse originated from Borena, Arsi, Bale, Hararge, and mainly Nazareth and its surroundings. The cattle for Debre Zeit originated from Nazareth and mainly Debre Zeit and its surroundings. The animals were usually brought to Addis Ababa market on foot or by truck. They were purchased by restaurant owners and taken to Addis Ababa slaughterhouse for slaughter. The animals are slaughtered after antemortem examination within 1 to 6 hours of their arrival to the slaughterhouse. In this slaughterhouse an average of 250 sheep,75 goats and 700 heads of cattle are slaughtered daily. In addition about 50 swine are slaughtered per week. It has separate slaughtering sections for small ruminants swine and cattle. It has about 700 man power. In case of Debre Zeit and Nazareth slaughterhouses, an average of 45 head of cattle were slaughtered daily.

3.3. Study design

A cross-sectional study was under taken in Addis Ababa, Debre Zeit and Nazareth slaughterhouses from October 2006 to May 2007. On each sampling day usually once a week 10-20 animals were randomly selected and sampled from amongst apparently healthy slaughtered animals. Kidney, and muscle samples were aseptically collected from each animal in separate sterile sample containers and transported in ice box packed with ice to the laboratory.

The samples were stored at freezer temperature (-20 ^{o}c) before analysis. All samples were processed and analyzed separately.

3.4. Sampling

Individual animals to be sampled were selected using random sampling technique. From each selected slaughter cattle, kidney and muscle samples of 50-100 g were aseptically collected. The qualitative tests and quantitative determination was under taken at the Ethiopian Drug Administration and Quality control Laboratory (DACA).

3.4.1. Sample size estimation

The sample size required for the study were determined on the expected occurrence (prevalence) of drug residue and desired absolute precision according to Thrusfield (1995) by the following formula.

$$N=\frac{1.96^2 P_{exp}(1-P_{exp})}{d^2}$$

Where :N=required sample size

P exp= expected prevalence

d = desired absolute precision

Using a 95% confidence level, 5% absolute precision and 50% expected occurrence of drug residues in slaughtered animals, the sample size for this study was 384.

3.4.2. Sampling procedures

On each sampling day 10-20 randomly selected cattle (2 types of samples from each animal =20-40 samples) were sampled. Approximately 50 to 100 grams of kidney or muscle samples obtained from each carcass. Each sample was labeled and wrapped in polythene bags and put in cool boxes with dry ice or freezer packs at 4^{0}C. The samples were then transported to the laboratory and stored at -20 °C until time of analysis.

3.5. Methods for tetracycline residue analysis

The chemicals, reagents, apparatus and detailed procedure are presented fully in Annex 1 and 3

3.5.1. Detection of tetracycline residue in meat using thin layer chromatography (TLC)

TLC method was used as a screening test. Thin layer chromatography is a sensitive and exact method for monitoring low amounts of different biological and chemicals. Illumination of tetracyclines against UV light helps as a simple detector for the analysis. In this study TLC was used for the detection of tetracycline residue following the techniques recommended by Tajick and Shohreh (2006).

Tetracycline extraction

10 gram of ground kidney tissues of cattle in 10 ml 96% ethanol was crashed and squeezed fine in a Chinese mortar. The solvent was transferred to 15 ml falcon centrifuge tubes and centrifuged at 4000 rpm for 10 minutes. The clear supernatant transferred to clean glass test tubes and evaporated in water bath at 80 °c. After full drying the deposits resolved in 0.2 ml methanol. The samples were ready to point on silica plates (Tajick *et al.,* 2002).

Preparation of silica plates

Glass plates (10 × 20 cm dimensions) were washed in acetone bath. For each plate 2 g of silica gel 60 plates (Merck, Germany) mixed in 5 ml distilled water and shaked thoroughly to produce fine paste. Clean glass plates were coated with silica paste by TLC gel spreader system (CAMAG, USA) in 0.25 mm thickness. Plates were activated in 120 ^{o}C for two hours (Boyer, 1993).The pH of disodium edetate were adjusted to 7.0 by a 10% w/v solution of 10 molar sodium hydroxide and sprayed evenly onto the plates (about 10 ml for a plate of 100 x 200 mm) The plates were then allowed to dry horizontally for at least 1 hour. Before use the plates were allowed to dry at 110 °c for an hour (British Pharmacopia,1999).

*Standard preparation***:** For comparison of extracted residues with raw antibiotics, reference standard (Sigma Chemical Co., St. Louis MO. USA, supplied analytical standards of

oxytetracycline, tetracycline and Doxycycline) of 1µg/ml was dissolved in methanol (Thangadu *et al.*, 2002).

Pointing, running and detection

About 10 µl of methanol dissolved deposits were pointed on silica plates treated plates transferred to TLC tank containing a saturated mixture of dichloromethane, methanol and water (59: 35: 6 by volume) as mobile phase. After receiving of solvent front to end of plates they were removed off and dried in a current air and examined under ultraviolet light at 366 nm. (British Pharmacopia,1999; Tajick *et al.,* 2002).

The results were evaluated depending on the purpose of a chromatographic analysis. For qualitative determination localization of substances was sufficient. This could be easily achieved by parallel runs with reference substances and the color of the spot. A parameter used for qualitative evaluation was the retention factor (Rf value) and the color of the spot. The sample with Rf equals to and similar color of the spot with the reference standard was considered to be positive The Rf value is defined as follows:

$$Rf = \frac{\text{Distance from the starting line to the center of spot}}{\text{The entire distance moved by the solvent}}$$

3.5.2. Determination of tetracycline residue levels with high performance liquid chromatography (HPLC)

Samples that were previously considered as positive by thin layer chromatography were subsequently analyzed by high performance liquid chromatography following the standard of Agence Française de Sécurité Sanitaire des Aliments (AFSSA) for determination of tetracycline residues in kidney and muscle by high performance liquid chromatography.

Sample pretreatment

The samples were kept at -20 °C until analysis. Before analysis samples were allowed to defrost at room temperature. Each sample to be analyzed was ground into fine powder using sartorius mincer and 5 g was weighed using a balance in to a centrifuge tube. 25 ml McIlvaine buffer-EDTA solution was added to the tube and was blended 30 seconds by shaking and then tube

vortex-mixed for 15 minutes and centrifuged 10 min at 4000 g about 4°C.The supernatant was transferred to a beaker on a magnetic stirrer and 2.5 ml trichloroacetic acid was added slowly with constant stirring. It was centrifuged again for 5 minutes at 3000 g. Then single GF/B filter paper was fixed in buchner funnel moisturized with McIlvaine buffer-EDTA. And the supernatant was filtered through funnel.

Sample clean up by solid phase extraction

Solid phase extraction (SPE) cartridge was conditioned with 1ml methanol,1ml McIlvaine buffer solution and 1 ml of HPLC grade water. The final extract was applied onto the cartridge. When the extract loading completed, tetracycline was eluted with 1 ml of 0.01molar oxalic acid in methanol and next with 1ml HPLC grade water. Determination of the tetracycline residues was done using a high-pressure liquid chromatography (model Shimadzu Class-VP Series, Kyoto, Japan) equipped with SIL-10 autoinjector with sample cooler and LC-10 on-line vacuum degassing solvent delivery unit. Chromatographic control, data collection and processing were carried out using Shimadzu Class VP data software, a constant flow pump and a variation wavelength UV detector set at 355 nm was used for analyzing data. The separation was done on Nucleosil C18 (5µm, 250x 4.0 mm I.D.E Merck) column with acetonitrile and 0.01 M aqueous oxalic acid solution by gradient mode as the mobile phase flow-rate of 0.8 ml/min at room temperature and the sensitivity range was 0.08 ppm. HPLC analysis was performed in 20 minutes.

Preparation of standard stock and working solution

Sigma Chemical Co., St. Louis MO. USA, supplied analytical standards of oxytetracycline and tetracycline and Doxycycline. Stock solution of 100µg of tetracyclines were prepared by diluting 100 mg of reference standard to 100ml with methanol. The intermediate solution of 100 µg/ml was prepared by diluting of 10 ml of stock solution to 100 ml with methanol. The working solution of 1 µg/ml was prepared by diluting 50 µl of 100 µg/ml solution to 5 ml methanol. Standard solutions were prepared from 0.125 to 1 µg/ml and from 0.75 to 6 µg/ml for muscle and kidney respectively. (These solution were kept in -20^{o}C).

Interpretation of results

Tetracyclines were detected by UV absorption at 355 nm. A given sample was regarded as positive for tetracycline if its retention time (from the time a substance is injected until it emerges from the column and passes through the detector) and peak corresponded to that of the standard. Results for the positive samples were plotted automatically on the integrator. Their corresponding areas were recorded only if the retention time were equal to 10.5, 12.75 and 18.9 minutes for oxytetracycline, tetracycline and doxycycline respectively with 2% deviation This was done in triplicates for the samples. Since the concentration of standard was known, calculations to get the concentration of the samples were extrapolated from the calibration curve, taking into account the calculated recovery (Annex 2). The detailed procedures are mentioned in the standard of Agence Francaise de Securite Sanitaire des Aliments (AFSSA) for "Determination of tetracycline residue in kidney and muscle by high performance liquid chromatography"(Annex 1).

3.7. Data management and analysis

The data was entered and managed in MS Excel and SPSS software programs. Intercooled Stata version 7.0, 2001 and SPSS version 11.5, 2000 soft wares were applied for the data analysis. Descriptive statistics such as percentages and frequency distributions were used to describe/present the nature and the characteristics of the data.

4. RESULTS

A cross sectional study was conducted on apparently healthy slaughtered cattle at Addis Ababa, Debre Zeit and Nazareth slaughterhouses (n=384) from October 2006 to May 2007 to detect and determine tetracycline residue levels in kidney and muscle samples of cattle.

4.1. Results of the qualitative analysis (TLC)

Analysis of kidney with TLC showed that majority of the samples have variable amounts of oxytetracycline. Concentration of extracted liquid was an important stage in monitoring of residue. Pointing of centrifuged solvent on silica plates after concentration made detection of tetracycline easy and easier. Similarities between retention factor (Rf) i.e. distance from base line to the center of zone divided by distance from base line to solvent front (the entire distance moved by the solvent) of detected patches from samples with standards led us to be sure that there are correlations between them which is the characteristic for that compound under a particular chromatographic conditions. In this case Rf value was 0.32 (figure 3, 4 and 5).

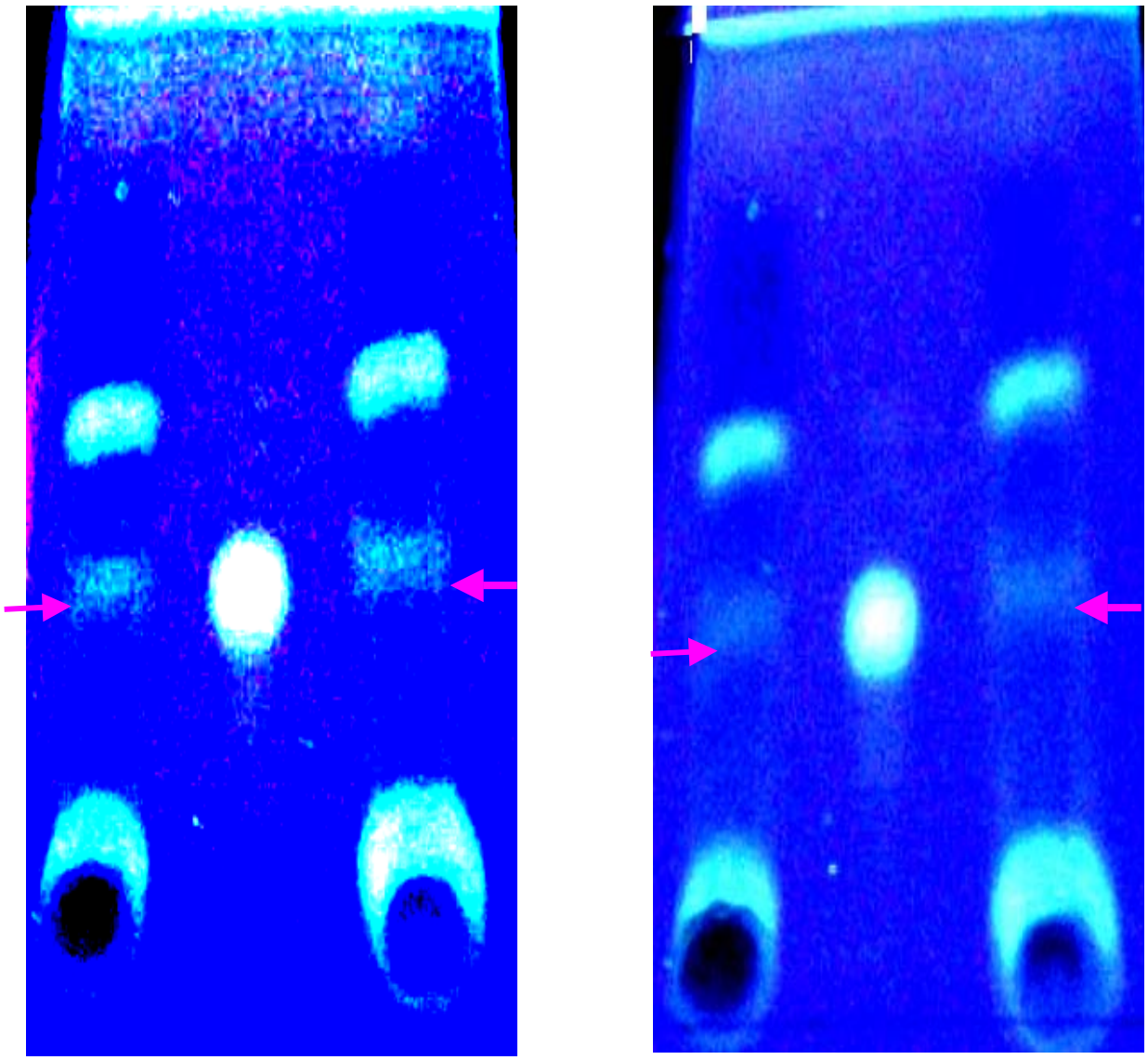


Figure 3. Samples positive under UV illumination by TLC The right and the lefts are samples and the center is reference standard The arrow indicates spot of oxytetracycline (Addis Ababa slaughterhouse)

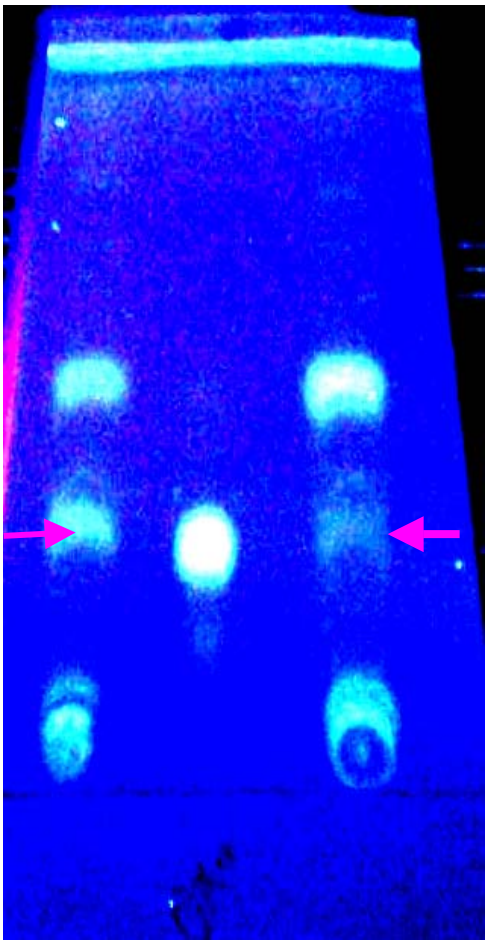


Figure 4. A picture taken from samples positive for oxytetracycline. The right and the lefts are samples and the center is reference standard (Nazareth slaughterhouse)

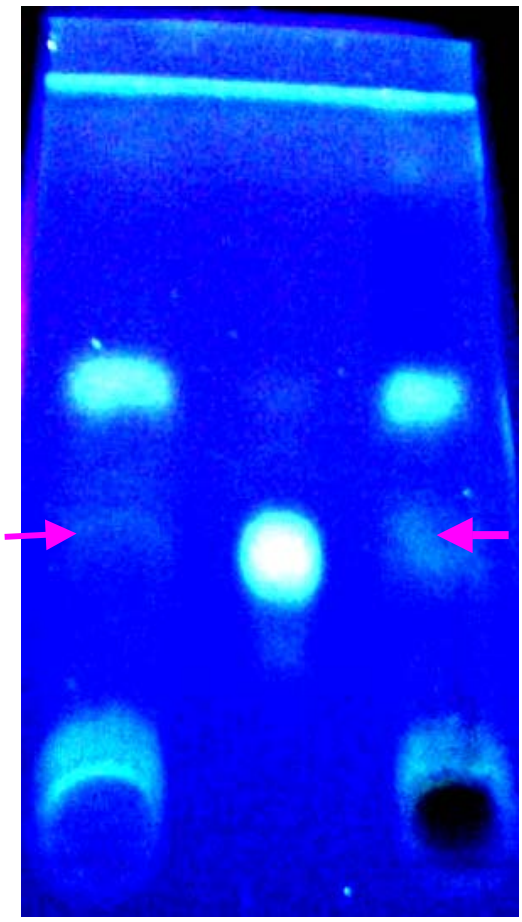


Figure 5. A picture taken from samples positive for oxytetracycline. The right and the lefts are samples and the center is reference standard (Debre Zeit slaughterhouse).

Out of the total 384 kidney samples analyzed during this study 274 (71.35%**)** had detectable levels for oxytetracycline residues. Tetracycline and doxycycline were not detected. In every sample where kidney sample had been positive for oxytetracycline by TLC, muscle samples were also positive by HPLC. Numbers of the samples (kidney and muscles) positive for oxytetracycline residues are shown in figure 6.

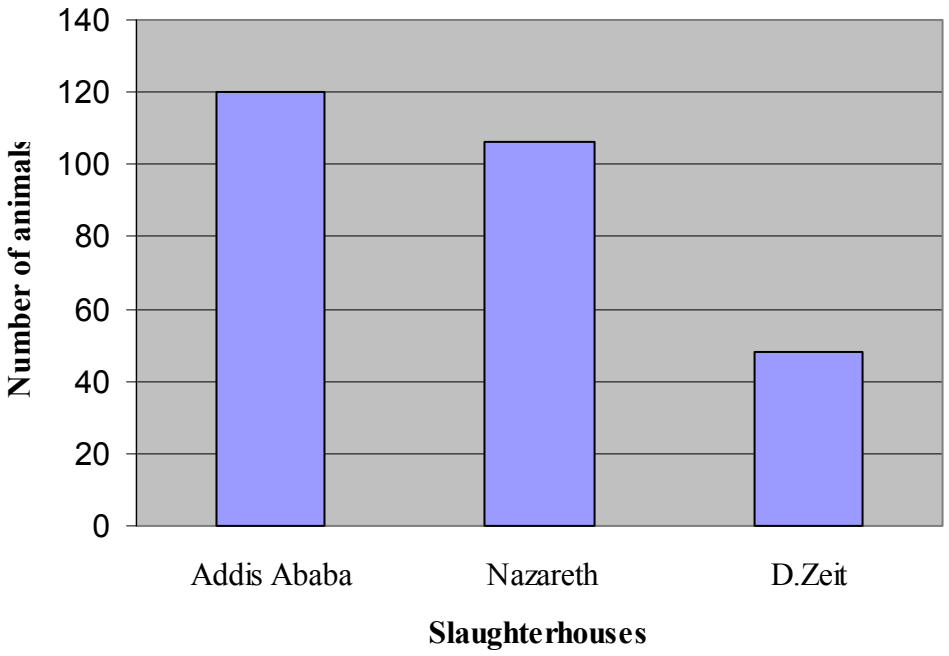


Figure 6. Number of animals positive for oxytetracycline residues in Addis Ababa, Debre Zeit and Nazareth slaughterhouses.

In Addis Ababa slaughterhouse 93.8% of kidney and beef samples were positive for oxytetracycline. In Debre Zeit 37.5% of kidney and beef were also positive for oxytetracycline while from Nazareth slaughterhouse 82.81% of kidney and beef samples were positive for oxytetracycline.

4.2. Results of the quantitative analysis by high performance liquid chromatography (HPLC)

The samples positive for thin layer chromatography were further analyzed by HPLC for quantification. The results of this study indicated that oxytetracycline residues previously detected in TLC were detected in all samples by HPLC. A given sample was regarded as positive for tetracycline if its retention time and peak corresponded to that of the standard. Retention time was considered a reasonably unique identifying characteristic of a given analyte. Figure 7 and 8 shows chromatograms (the visual output of the chromatograph) in which x-axis is the retention time and the y-axis is a signal obtained by UV diode array detector corresponding to the amount of oxytetracycline existing in the system. The peaks are characteristic of their identity, with a distribution around the mean position (apex of the peak) that is characteristic of the kinetic properties of the chromatographic system. The area inscribed by the peak is proportional to the amount of substance separated in the chromatographic system. To get the concentration of a oxytetracycline sample, a reference standard of a known concentration had been injected into the HPLC and concentration of the sample was extrapolated from the curves peak area.

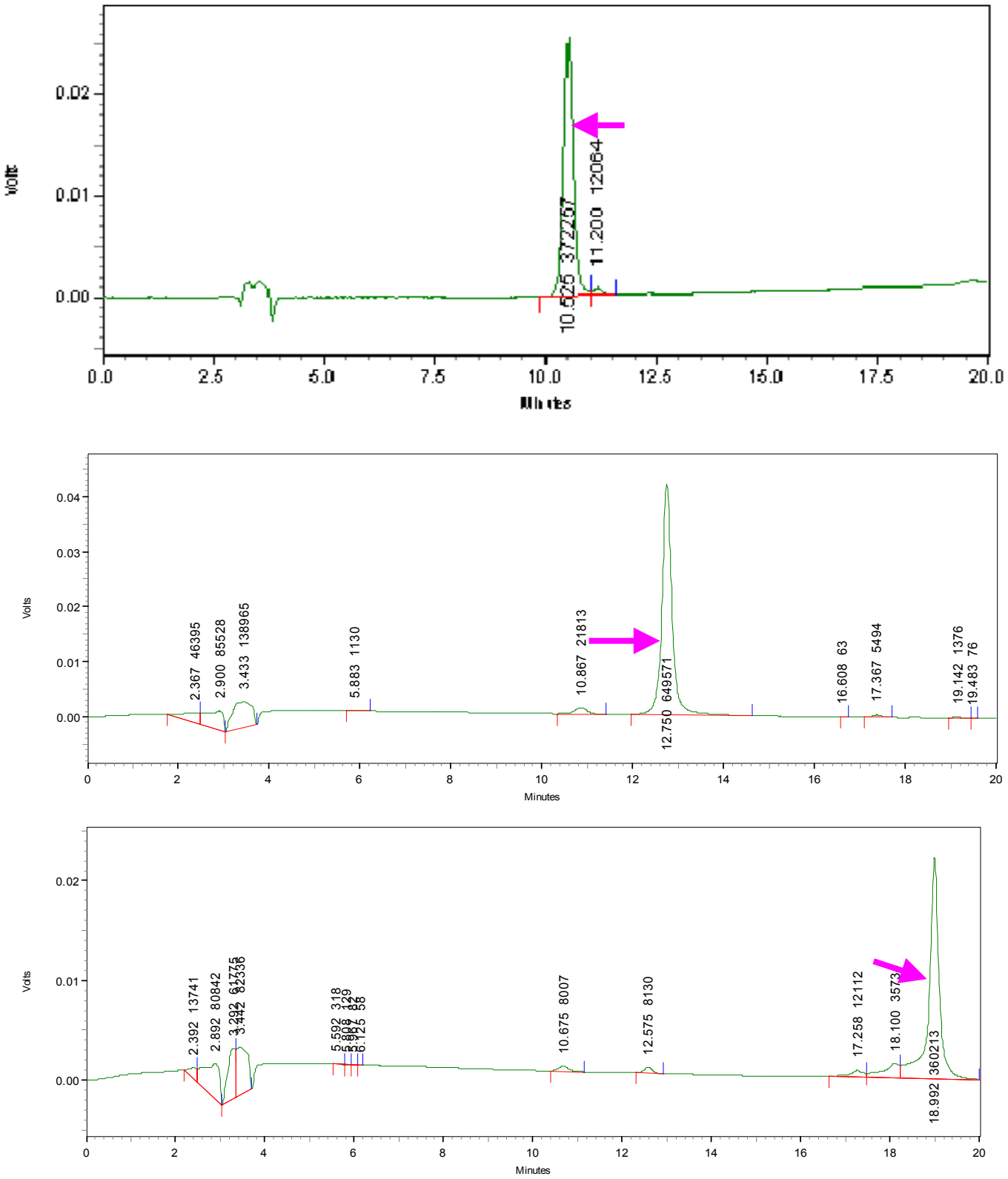
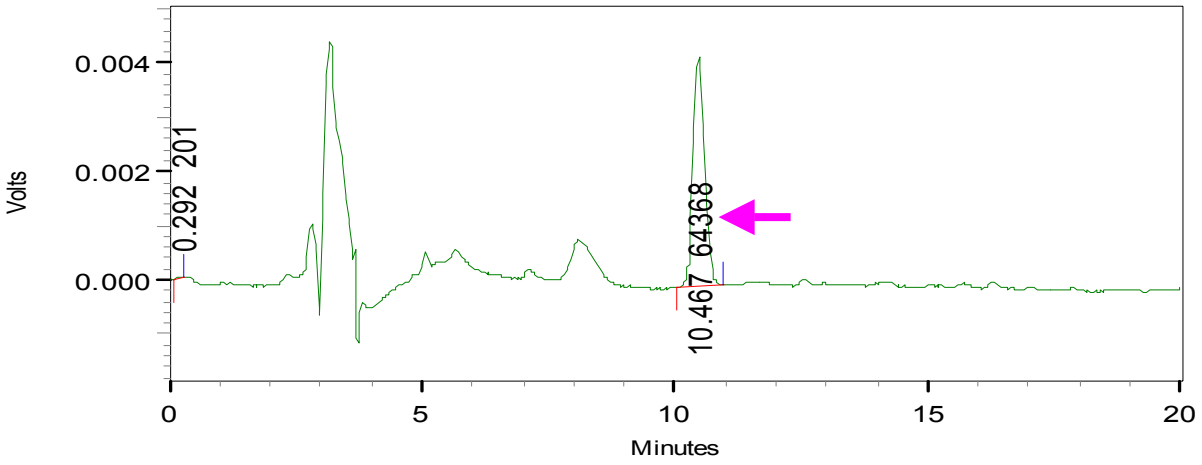


Figure 7. Chromatograms of reference standards: oxytetracycline, doxycycline and tetracycline (from top to the bottom). The arrow indicates the peak, peak area and its retention time.

A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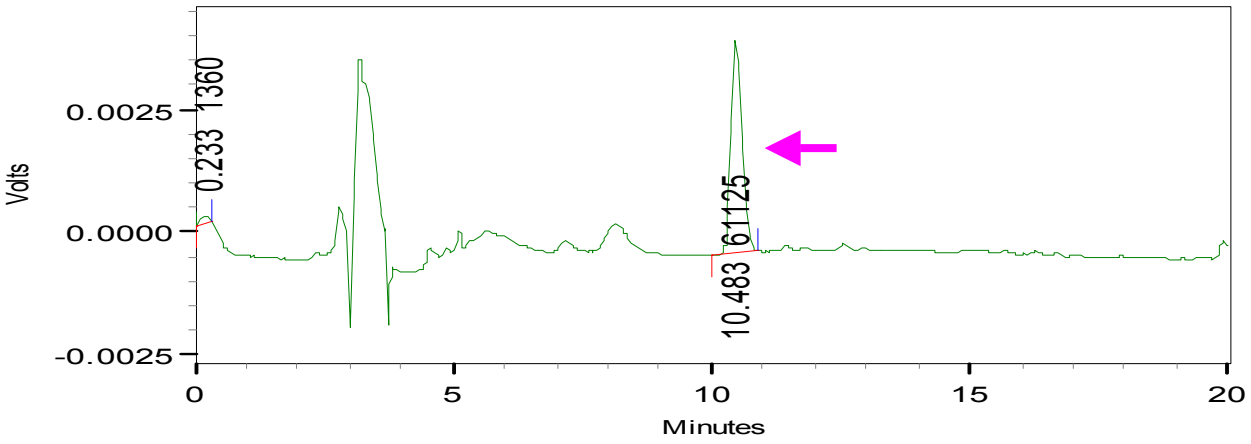
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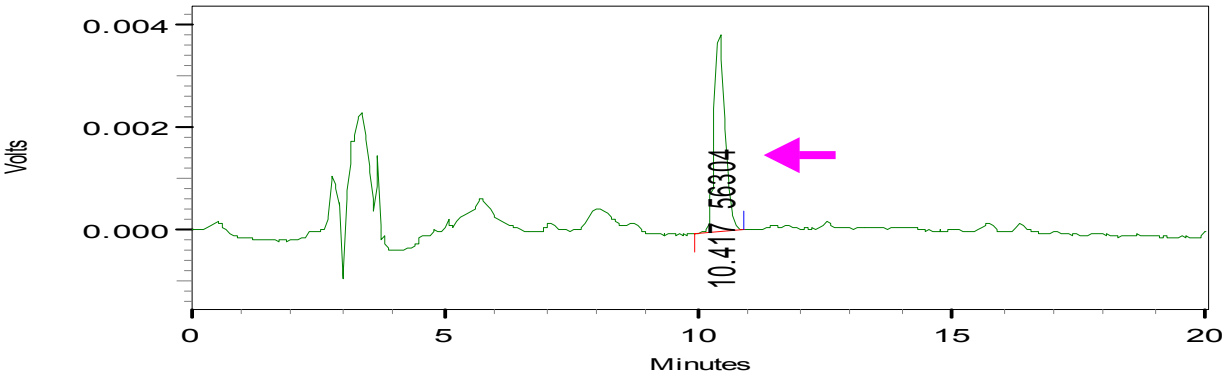
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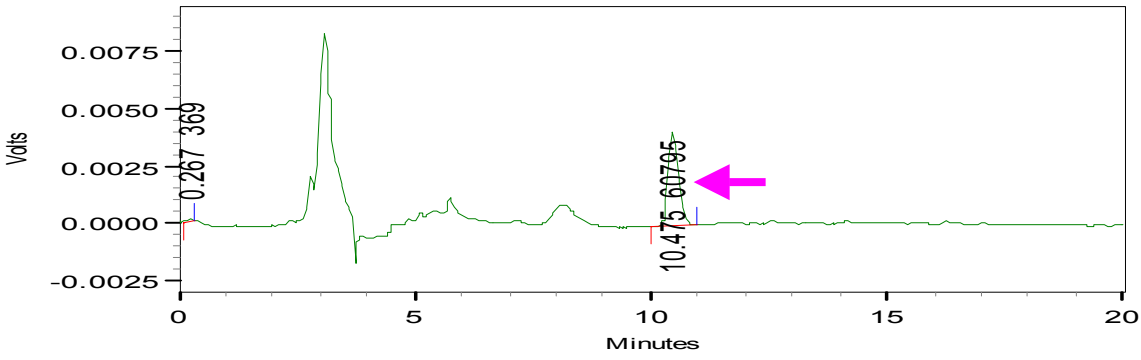
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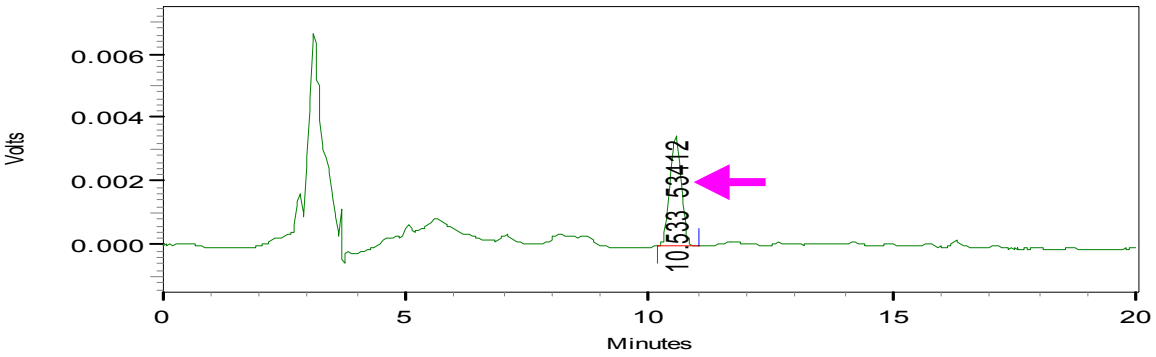
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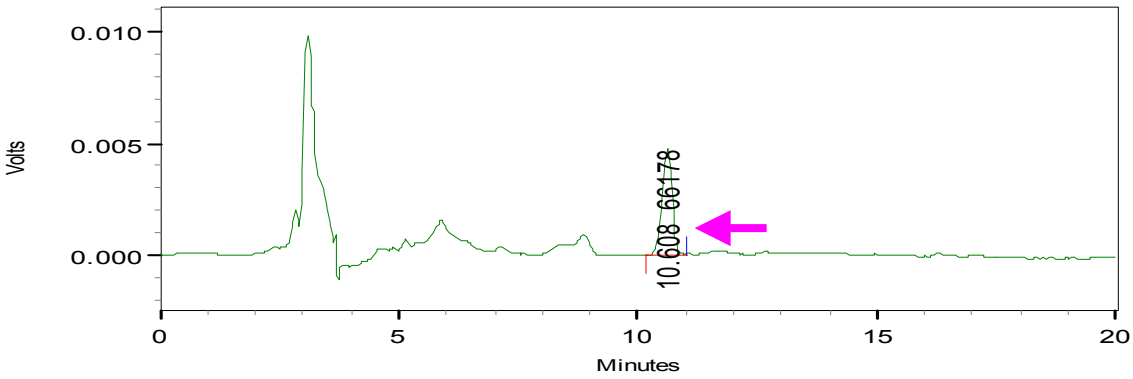
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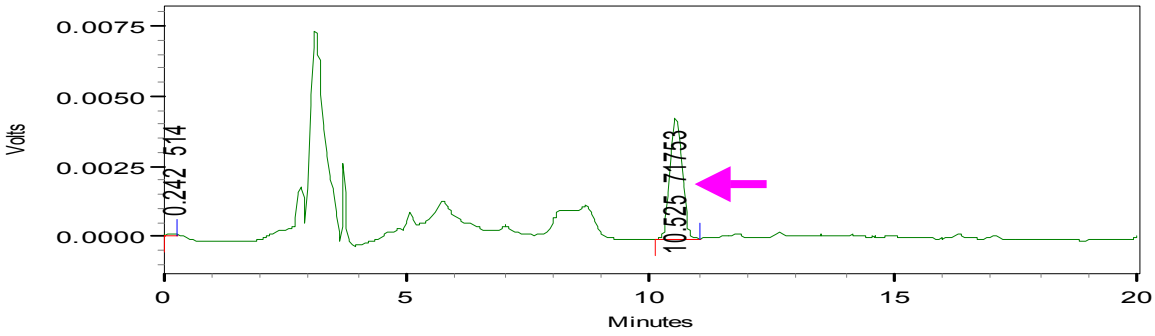
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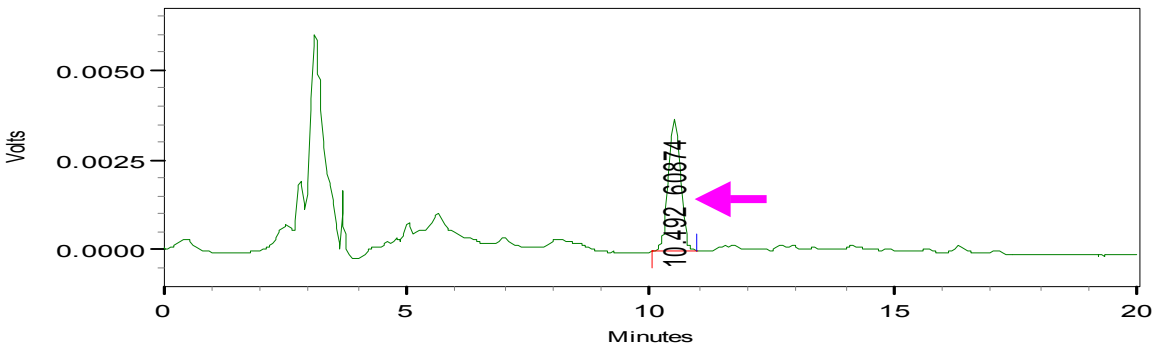
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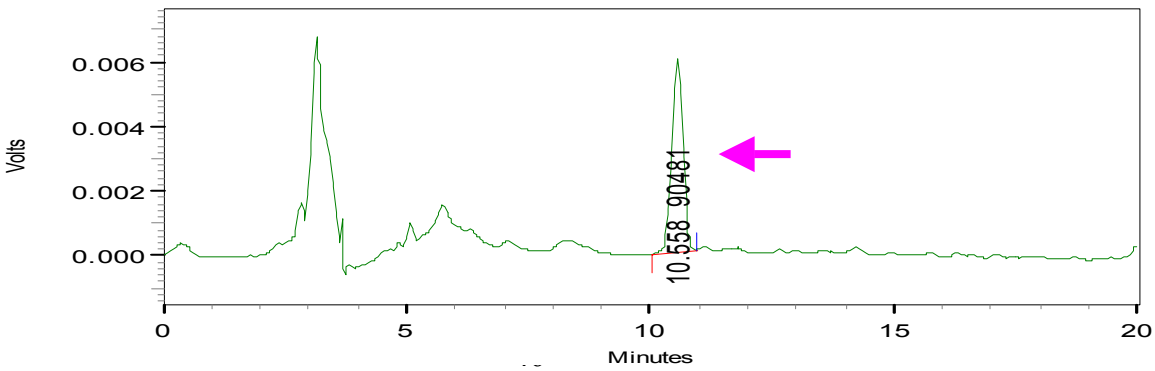
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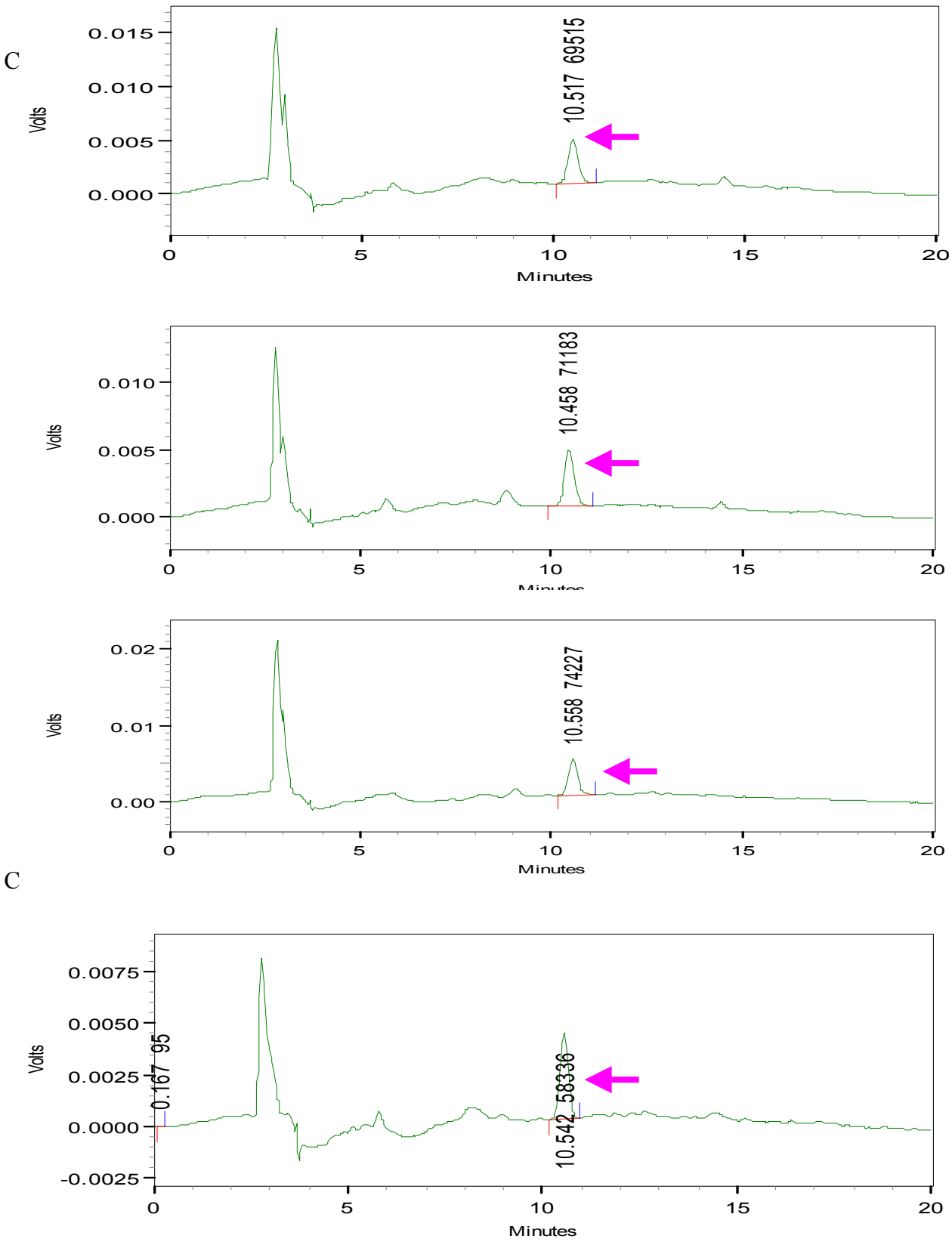

Figure 8. Chromatograms of some samples that were positive for oxytetracycline. A- Addis Ababa, B- Debre Zeit and C- Nazareth slaughterhouses. Arrow indicates the peak, peak area and retention time. C- from meat and others were form kidney samples.

The ranges for tetracycline residue levels from individual organs were: 9.732µg/Kg to 449.65µg/Kg for kidney, and 11.5µg/Kg to 429.289µg/Kg for muscle at Addis Ababa Slaughterhouse; 56.037µg/Kg to 740.59µg/Kg for kidney and 3.01µg/Kg to 145.87µg/Kg for muscle at Nazareth slaughterhouse and 63.12µg/Kg to 260.56µg/Kg in kidney and 6.68µg/Kg to 67.34µg/Kg in the muscle at Debre Zeit slaughterhouse.

Mean oxytetracycline residue levels in muscle from the three slaughterhouses were not significantly different ($p>0.05$) but oxytetracycline residue levels in kidney were significantly different ($p<0.05$). The mean, range and numbers of the samples (kidney and muscles) positive for oxytetracycline residues are shown in Table 6. Categories of different concentration are shown in figure 8.

Table 6. The mean, range and numbers of the samples (kidney, and muscles) positive for oxytetracycline residues

Area of study (slaughter houses)	Tissue types		
		Beef	Kidney
Addis Ababa	Positive	120/128	120/128
	Mean	108.349	102.49
	Range	11.5- 429.3	9.73- 449.6
	Std. deviation	61.321	49.67
Nazareth	Positive	106/128	106/128
	Mean	64.852	113.18
	Range	6.7- 67.3	63.1- 260.6
	Std. deviation	35.469	69.88
Debre Zeit	Positive	48/128	48/128
	Mean	15.916	116.53
	Range	3.0- 145.8	56.0- 740.6
	Std. deviation	12.288	34.02

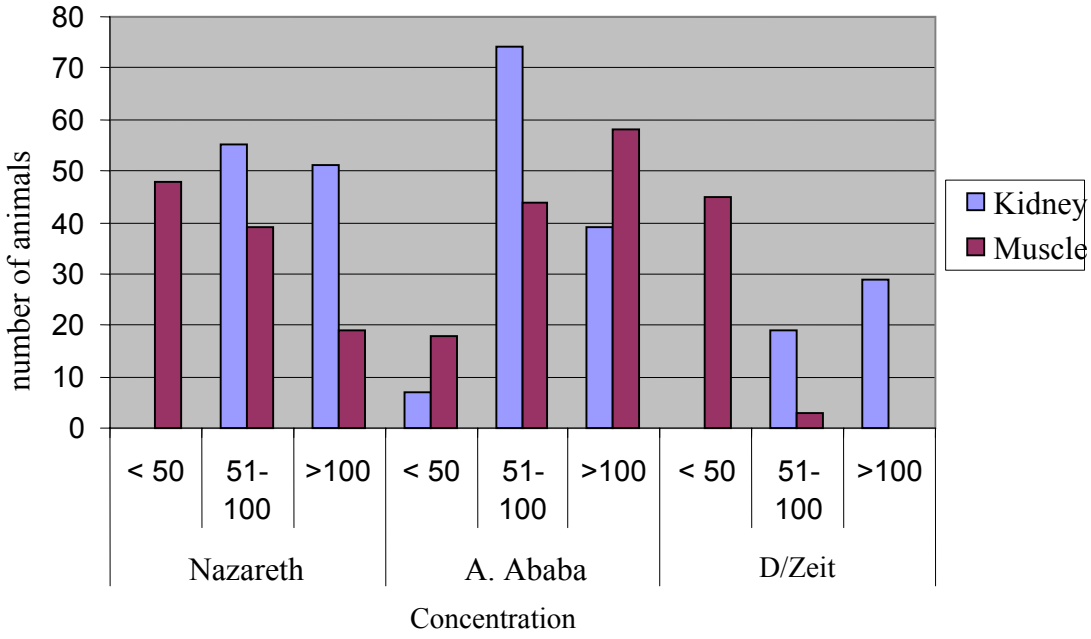

Figure 9. Categorical representation of oxytetracycline residue concentrations in animals slaughtered in the three slaughterhouses

The oxytetracycline positive samples which showed residues of oxytetracycline above MRLs were 58 (48.33%) at Addis Ababa slaughterhouse and 51 (48.11%) at Nazareth slaughterhouse in muscles and 1 (0.9%) in kidney samples at Nazareth slaughterhouse. At Debre Zeit slaughterhouse no samples were above the maximum residue limit. Figure 9 displays the mean detectable concentrations of oxytetracycline in muscle in comparison with the recommended maximum residue limit (MRL) for oxytetracycline (100µg/kg) in Addis Ababa slaughterhouse. Figure 10 illustrates the mean detectable concentration of oxytetracycline in muscle in comparison with the recommended maximum residue limit in Nazareth slaughterhouse. Figure 11 illustrates the mean detectable concentration of oxytetracycline in muscle comparison with the recommended maximum residue limit in Debre Zeit slaughterhouse.

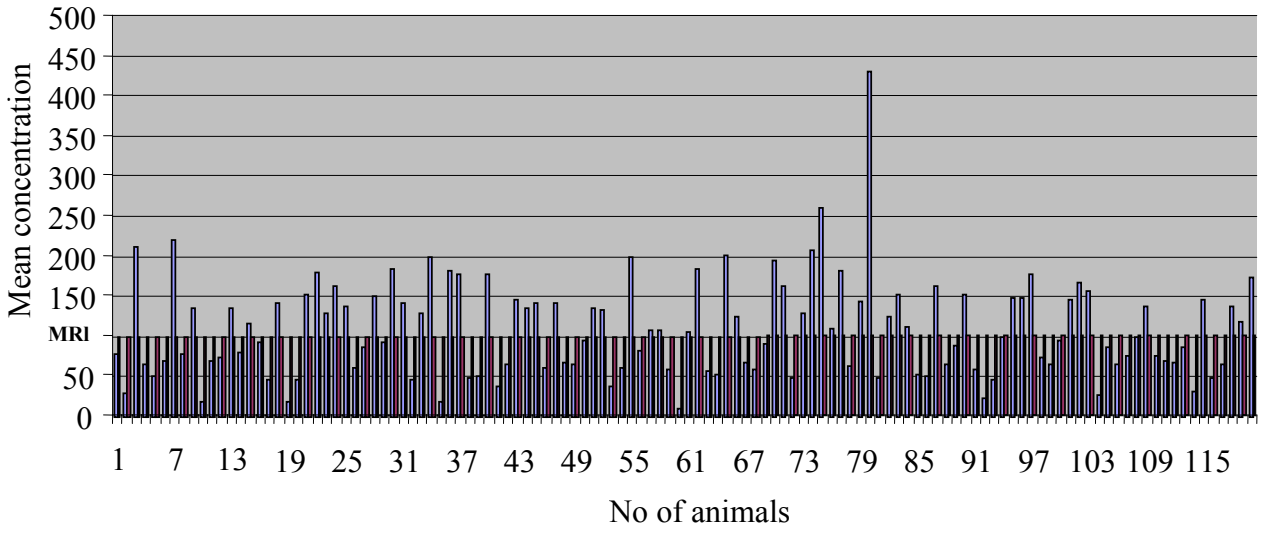

Figure 10. Mean detectable concentrations (µg/kg) of oxytetracycline in muscle samples in comparison with the maximum residue limit (MRL=100 µg/kg) (Addis Ababa slaughterhouse)

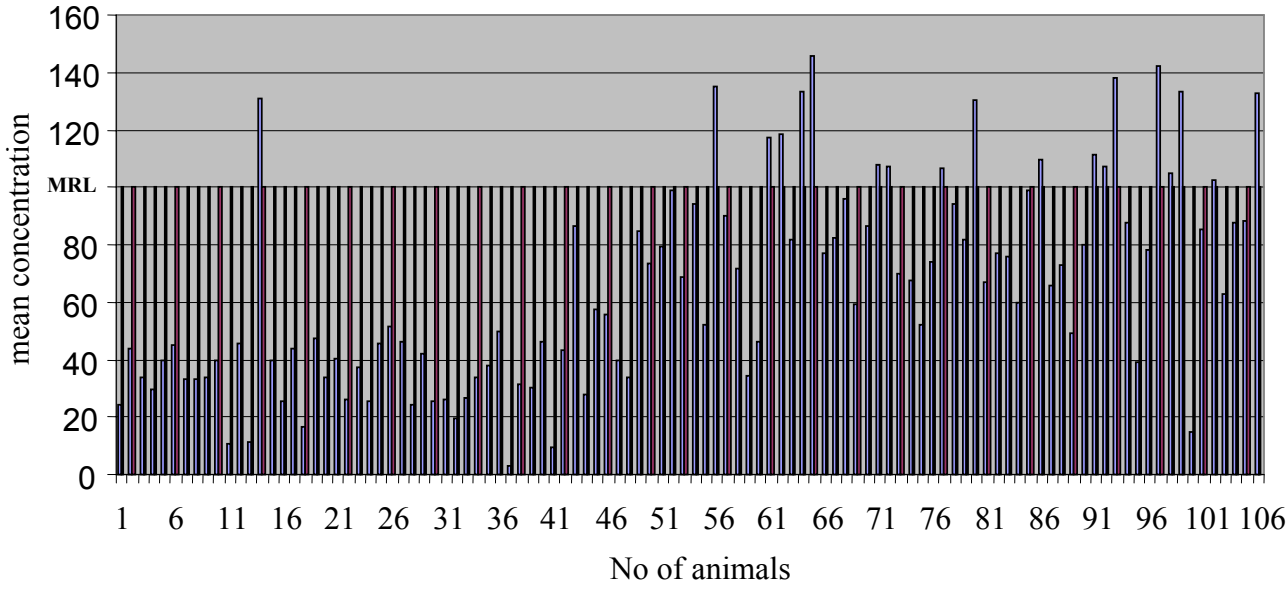

Figure 11. Mean detectable concentrations (µg/kg) of oxytetracycline in muscle samples in comparison with the maximum residue limit (MRL=100µ/kg) (Nazareth slaughterhouse)

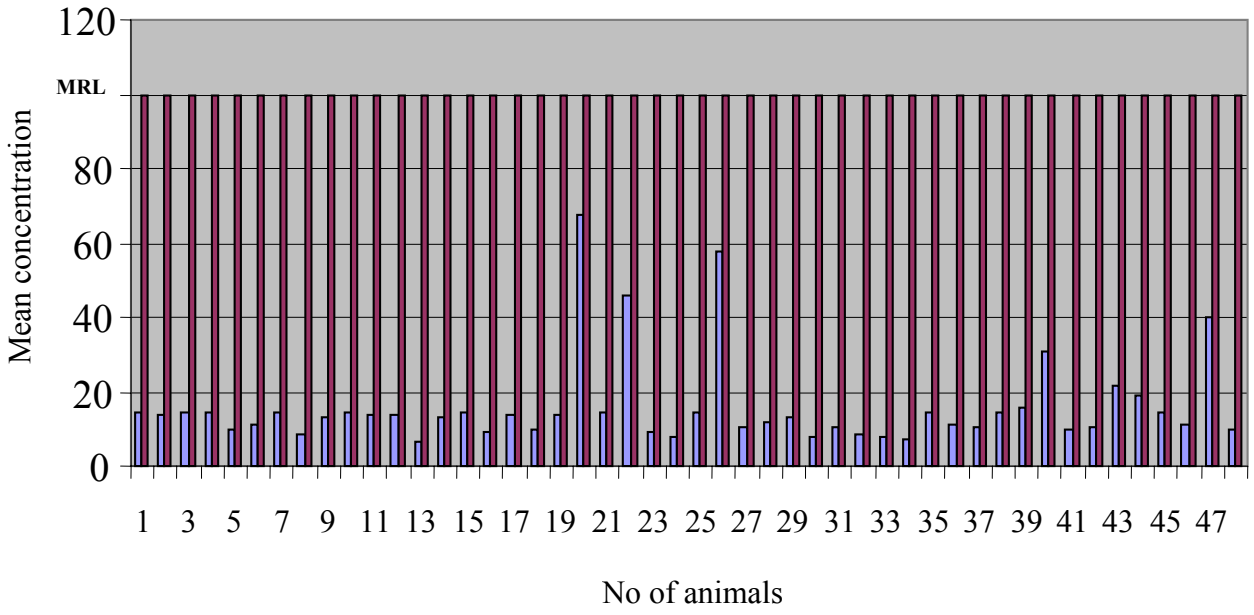

Figure 12. Mean detectable concentrations (µg/kg) of oxytetracycline in muscle samples in comparison with the maximum residue limit (MRL=100µ/kg) (Debre Zeit) slaughterhouse)

5. DISCUSSION

5.1. Proportion of tetracycline antibiotic residues

Out of the total 384 meat samples analyzed during this study, 274 (71.35%) had detectable levels for oxytetracycline residues. Tetracycline and doxycycline were not detected in beef and kidney samples. The proportion of oxytetracycline positive samples were much higher as compared to other reports elsewhere. Results of the FSIS-USDA National Residue Program for combined meat and poultry samples in USA (FSIS-USDA, 1991; 1992; 1993; 1994; 1995; 1996; 1997) revealed that, among monitoring samples in 1991, 0.26% showed violative concentrations of chemical residues; comparable data for 1992 (0.29%), 1993 (0.26%), 1994 (0.18%), 1995 (0.21%), 1996 (0.16%) and 1997 (0.27%) suggest that the average annual incidence, during this decade, of violative chemical residues in meat and poultry tissues is about 0.23% (1 in 435). It was also reported that in western countries such as the UK (Gracey *et a*l.,1999) 44 out of over 12,300 samples collected from pigs contain chlortetracycline and in Ireland with 42% and 12% pork samples were positive to chlortetracycline for the period of 1996/1997 and 1997/1998, respectively (TEAGASC, 2001).

In the study undertaken in Vietnam (Duong, 2005) it was also indicated that 5.52% pork samples were positive for tetracycline residues which were much lower than the present study. A similar study carried out by Muriuki *et al*. (2001) in Nairobi and surrounding areas reported that out of a total of 250 beef samples analyzed during this study 114 (45.6%) had detectable levels for tetracycline. Another study conducted in Iran also indicated that (Rahim *et al.,*2006) samples from edible tissues of 86 (95.55%) of samples of chickens of broiler farms in Tehran, had residues of oxytetracycline which are comparable with the results of this study.

To date, no any study on the prevalence of tetracyclines and other veterinary drug residues in food animal and food of animal origin in the country has been reported which can show the magnitude of the problem and enable the public to chose or buy meat and milk products without antibiotic residue. The results obtained confirmed that oxytetracycline was heavily and indiscriminately used in the fattening farms, small scale fattening farms, pastoralists and other animal owners. The results of this study also suggest that the recommended withdrawal time was

either not strictly applied or extra label treatment of this drug might be used. The possible explanations for this might be:

i. The people think that oxytetracycline and albendazole are the basic drugs which are highly essential for fattening their animals and they are indiscriminately given to animals when they want their animals to eat more (appetizers) and put on weight with short period of time to reach for marketing.

ii. Once animals got sick, farmers buy veterinary products from the market sold by informal vendors and administer to the animals without any veterinary prescription and diagnosis. Similar situation was reported in Vietnam and Kenya (Duong, 2005; Muriuki *et al*., 2001).

iii. The cattle slaughtered at Addis Ababa and Nazareth slaughterhouses originated from different parts of the country and mainly transported on their foot moving several kilometers. As a result of this, animals are subjected to transportation stress, the owners think that the animals need to be treated by the most available drugs such as oxytetracycline.

iv. Low level of control from the controlling authorities and information on the prudent use of veterinary drug is not available.

v**.** Lack of awareness of the risks associated with antibiotic residues due to absence of comprehensive studies on this issue at all levels in the country.

vi**.** Illegal veterinary drug trade.

5.2. Concentration of tetracycline residue levels and food safety

All beef samples had detectable level of oxytetracycline residue by HPLC analysis in which the relevant kidney samples had been positive for oxytetracycline by thin layer chromatography FAO/WHO Expert committee on Food Additives established MRL for oxytetracycline of 600 µg/kg in kidney;300 µg/kg in liver; 100 µg/kg in muscle (EMEA,1995). This study revealed that out of the 274 samples positive for oxytetracycline residues, 58 (48.3%) and 51 (48.1%) beef

samples in Addis Ababa and Nazareth slaughterhouses, and 1 (0.9%) kidney samples of Nazareth slaughterhouse had residues of oxytetracycline above MRLs. Similar study in Kenya also showed that 20% of Athi River slaughter beef had antibiotics and 55% of the beef from Dagoretti, Kiserian, and Dandora had violative residues of veterinary drugs (Mdachi *et al*.,1991). The number of samples above MRLs for tetracyclines was higher than that obtained in most countries in which such studies have been reported (Ryan and Dupont, 1974 Tittger *et al*.,1980). The present study is also higher than the study conducted in Iran (Rahim *et al.,*2006) which indicated that the oxytetracycline positive samples, which showed residues of oxytetracycline above MRLs, were 25 (27,77%), and 17 (18.88%) in muscles and kidney samples respectively.

The use of antimicrobial agents in food animals has caused concern regarding the impact these uses have on human health. Use of antimicrobial agents in animals and humans results in the emergence and dissemination of resistant bacteria. Resistant bacteria from food animals may be passed through the food chain to human resulting in resistant infections. Increasing resistance to antimicrobial agents that are important in the treatment of human diseases, such as tetracyclines fluoroquinolones and third-generation cephalosporins for the treatment of *Salmonella* and *Campylobacter* infections, has significant public health implications (Alicia *et al.,*2003*).* The study also showed that oxytetracycline is imprudently used in the country which is the basic means for the above problem and one can say that tetracycline will be out of use due to treatment failures. The development of resistance to *Salmonella*, was reported from Ethiopia by several authors (Molla *et al*., 2003,Alemayehu *et al*., 2003 and Zewdu,2004) and other African countries (Gedebu and Tassew, 1981; Adesiyun and Oni, 1989; Leegard *et al*.,1996).

According to Zewdu (2004) investigations on antimicrobial resistance of Salmonella isolates from food of animal origin and personnel in Addis Ababa, 32.7% were resistant to many antimicrobials including tetracyclines. He also indicated that from 32 resistant Salmonella isolates chicken samples accounted for 56.35 while pork, mutton, minced beef and cottage cheese accounted for 21.9%, 9.4%,9.4% and 3.1% respectively. The high prevalence of *Salmonella* isolates resistant to these relatively cheaper and commonly available antimicrobials is disturbing because of the limited access and high cost of newer cephalosporins and quinolone drugs (D'Aoust,1989) for poor citizens of developing countries like Ethiopia. Furthermore, systemic

spread of such resistant isolates in human host could lead to serious complications or to a fatal out comes. On the other hand the occurrence of resistance to the quinolone and fluoroquinolone (ciprofloxacin) in 9.4% of resistant isolates from chicken carcass and minced beef or 3% of the total *Salmonella* isolates was indicated here in Ethiopia and found to be striking because development of resistance against these drugs undermines the value of this first line drugs for the treatment of human systemic salmonellosis (Zewdu,2004). The reasons for the emergence of resistance against these drugs were not known and deserves investigation. However, most likely, indiscriminate use of antimicrobials in the public heath sector and administration of sub therapeutic dose to animals for treatment and for prophylaxis or feed additives purposes could be some of the factors associated. Alicia *et al. (2003)* reported that the emergence of fluoroquinolone resistance among *Campylobacter and Salmonella* is an example of antimicrobial resistance resulting from the use of antimicrobial agents in food animals and the subsequent transfer via the food supply of resistant bacteria to humans.

In addition, human exposure to animal products containing significant level of antibiotic residues may prove immunological response in susceptible individuals and cause disorders of intestinal flora. Some individuals may have an allergic reaction to these compounds. As undesirable side effects, tetracyclines not only discolor the primary and permanent teeth but also causes hypoplasia in developing teeth when administered to infants, mothers during the last two trimesters of pregnancy and children under 12 years of age (Walton *et al*.,1994). It has also been suggested that discoloration caused by tetracyclines occurs in adult dentition (Tanase *et al.,*1998).

Drug residue remains very significant from the prospective of international trade and consumer confidence, because it results in international trade barrier. As tariffs are removed and goods flow freely between countries, importing countries must be in confident that goods available for purchase are safe, and in addition to this, from time to time, there is pressure to use antimicrobial residues on non tariffs barrier to import (Kanneene and Miller, 1997). Now a days Ethiopia is exporting livestock and livestock products to some Arabian countries earning considerable foreign currency annually. Furthermore there is a great need to export to the European and American countries in the coming few years but the requirement by the World Trade Organization (WTO) and Codex should be met to do so. One of the requirements is that

antimicrobial residues in food should be below MRLs. For example, laws of inspection requirements of the United state states (FDA,2001) that all carcasses, parts of carcasses, meat, and meat food products of cattle, sheep, swine, goats, horses, mules, or other equines, capable of use as human food, offered for importation into the United States shall be subject to the inspection, sanitary, quality, species verification, and residue standards applied to products produced in the United States. Any such imported meat articles that do not meet such standards shall not be permitted entry in to the United States. Each foreign country from which such meat articles are offered for importation into the United States shall obtain a certification issued by the Secretary stating that the country maintains a program using reliable analytical methods to ensure compliance with the United States standards for residues in such meat articles. This study confirmed that oxytetracycline was heavily and indiscriminately used in the fattening farms, small scale fattening farms, pastoralists and other animal owners. This indiscriminate use of veterinary drugs can hinder the country's interest to fulfill the need to export to those countries. Therefore, attempts should be made to reduce the magnitude of the problem at various levels through the prudent use of antimicrobials such as oxytetracycline and awareness need to be created at different levels including controlling authorities, concerned organizations and the consumers as well.

5.3. Assessment of the methods used

This study followed a regular procedure in the analysis of antibiotic residue: a screening followed by a confirmation test. For the screening purpose thin layer chromatography was used which is a sensitive, high degree of precision, cost effective and exact method for monitoring low amounts of different biological and chemicals. Illumination of antibiotics against UV light helps as a simple detector for this mean (Tajick and Shohreh, 2006). Several workers have used the high-pressure liquid chromatography analytical method for tetracyclines in various samples: beef (Youji *et al*., 1984) human serum (Nilsson *et al*., 1976) honey, (Diaz *et al*., 1990) and liver and kidneys (Terhune *et al*., 1989). Although they worked on different products one common finding in the use of the HPLC was that the method was accurate sensitive, precise, specific and convenient analytical method for the, detection and quantification of tetracycline residues in biological specimens. One of the major advantages over other microbiological method is that the lower

detection limit of about 0.05-0.1 ppm makes it a high precision instrument analytical method. The detection levels were very low which is indicative of high sensitivity. The recovery of tetracyclines in fortified tissues using HPLC may reach 90% with coefficients of variation of 1.8-7.5% and detection limit of 5/10 mg/kg (Mulders *et al*.,1989).

Despite limitations of methods used in this study, it is recommended that the microbiological inhibition screening test using *B. subtilis* ATTC 6633, *B .megaterium, B. stearothermophilus* or other important American type cell culture organisms with HPLC, gas chromatography or mass spectrometry should be used widely to test tetracyclines and other veterinary drug residues in beef, pork, poultry meat and mutton. In addition to this, instruments used for the analysis and trained man power on the required area is mandatory since analytical techniques is complement of reducing or eliminating drugs residue dangers that enables us to get safe, wholesome food of animal origin in the country.

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6. CONCLUSION AND RECOMMENDATIONS

As an industry, livestock production in most countries depends upon other chemicals to improve animals performance and to control disease, one result of this discrepancy has been the occasional adulterating of human food supply by chemical residues like, antimicrobials. Residues are now a topic of considerable concern in food safety

The occurrence of oxytetracycline residues in the muscle and kidney samples taken from the study slaughterhouses is significantly high (71.35%). The oxytetracycline positive muscle samples, which showed residues of oxytetracycline above MRLs, were 58 (48.33%) at Addis Ababa slaughterhouse and 51 (48.11%) at Nazareth slaughterhouse and these figures can represent a real public health hazard.

Microbiological and chemical analysis of food of animal origin for foodborne organisms and veterinary drug residues is paramount importance in ensuring the supply of safe wholesome and sound food for consumers. The information obtained in this cross-sectional survey coupled with other future studies at different sites can be used as a basis to undertake qualitative and quantitative risk assessment and provide indispensable information in designing, monitoring and surveillance programs as well as control programs which can protect the consumers and promote reputability of the Ethiopian meat industry for both national and international markets.

Based on these facts the following recommendations are forwarded:

- Information on the actual rational drug use pertaining to veterinary drug use is not available in Ethiopia. There is need to make available those reliable and standardized drug use information in the veterinary practices of the country and there should also be good recording systems both at farms and slaughter levels.
- This study suggests the widespread misuse of oxytetracycline in cattle and lack of implementation of recommended withdrawal times. There is a need for strict regulation by the controlling authorities for the use of antimicrobial drugs in livestock industry as well as

the inspection of cattle for residues prior and after slaughtering and ensure that these are implemented at all levels.

- In addition to the regulatory approaches, national authorities should undertake appropriate education programs for animal producers, extension personnel, veterinarian, and consumers to create awareness of the problems of drug residues caused by the misuses of veterinary drugs.
- This is a preliminary work on veterinary drug residues in Ethiopia, there must be conditions and facilities that enables other researchers to carryout an extensive work to determine the magnitude of the problem and safeguard the consumer from the various effects of veterinary drug residues.

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8. ANNEXES

Annex 1: Method for determination of tetracycline residues in meat

(According to the standard of Agence Française de Sécurité Sanitaire des Aliments (AFSSA) for "Determination of Tetracycline residues in kidney and muscle by high performance liquid chromatography")

1- Scope and field of application

The present method allows the determination of the residues of four compounds and their epimers, oxytetracycline (OTC) and epi-oxytetracycline (epi-OTC), tetracycline (TC) and epi-tetracycline (epi-TC), chlortetracycline (CTC) and epi-chlortetracycline (epi-CTC) and doxycycline (DC). It is suitable for pork and bovine kidney and muscle. The detection limits in kidney are 80µg/kg, 60µg/kg, 170µg/kg and 160µg/kg for OTC, TC, CTC and DC respectively and 8µg/kg, 9µg/kg, 15µg/kg and 12µg/kg for OTC, TC, CTC and DC in muscle according to the criteria of the decision 93/256/EEC. Fortified samples from 300µg/kg to 2400µg/kg in kidney and from 50µg/kg to 400µg/kg in muscle have been analyzed in accordance with the criteria of this decision. Maximum residue limits for the sum of each tetracycline and its epimer (excluding epi-doxycycline) have been set up at 600µg/kg in kidney and 100µg/kg in muscle.

2- Principle

There are three principal stages in the samples preparation:

-Homogenization and extraction of the sample residues by EDTA/McIlvain buffer,

Precipitation of proteins using trichloroacetic acid and filtration,

Cleanup on solid-phase extraction cartridges C_{18} and injection.

Tetracyclines are separated on a C_{18} stationary phase and detected by UV absorption at 355 nm. The amount of tetracycline is calculated by interpolation from a calibration curve determined for each of the four compounds, taking into account the calculated recovery.

3- Chemicals and reagents

Unless otherwise specified, all reagents are of analytical grade. Demineralized water is obtained from an ultra pure water system (Millipore).

3.1- Acetonitrile (Merck, Art. 1.14291).

3.2- Methanol (Merck, Art. 1.06009).

3.3- Trichloroacetic acid (Prolabo, Art. 20742-293): dissolve 50 g in 50 ml ultra pure water to obtain a 1g/ml solution.

3.4- Oxalic acid dehydrate (Prolabo, Art. 28582.291): dissolve 1.20 g in 1 l ultra pure water to obtain a 0.01 M solution. Filter through a 0.45 µm unit under vacuum (4.1.20).

3.5- 0.01 M oxalic acid solution in methanol: dissolve 1.26 g in 1 l methanol.

3.6- Citric acid monohydrate (Merck, Art. 244): dissolve 21 g in 1 l ultra pure water.

3.7- Disodium phosphate anhydrous (Merck, Art. 1.06586): dissolve 28.4 g in 1 l ultra pure water.

3.8- Disodium ethylenediaminetetraacetate dehydrate (EDTA), (Prolabo 20 302 293).

3.9- Mc Ilvain buffer: mix 1 l citric acid solution (3.6) with 625 ml disodium phosphate solution (3.7) and adjust pH to 4.0 ± 0.05 if necessary.

3.10- McIlvain buffer/ETDA solution: prepare a 0.1 M ETDA solution in McIlvain buffer (60.5 g ETDA in 1.625 l).

3.11- Mobile phase: acetonitrile (3.1) and 0.01 M oxalic acid (3.4) in a gradient mode.

3.12- Standards:

3.12.1- Oxytetracycline hydrochloride, potency (Pfizer).

3.12.2- Tetracycline hydrochloride (Virbac).

3.12.3- Chlortetracycline hydrochloride (Vetoquinol).

3.12.4- Doxycycline hyclate (Veprol).

3.12.5- Epi-oxytetracycline (Acros).

3.12.6- Epi-tetracycline (Acros).

3.12.7- Epi-chlortetracycline (Acros).

3.13- Stock solutions:

N.B.: because interferences may occur between some of the standards, they will not be injected simultaneously for the quantification. For example, chlortetracycline standard

contains tetracycline. Each stock solution will be prepared with two tetracyclines as here under.

3.13.1- OTC and CTC stock standard solution: prepare a methanolic solution containing 1 mg/ml of OTC and CTC (+ their respective epimer if these epimers are contained in the standard).

3.13.2- TC and DC stock standard solution: prepare a methanolic solution containing 1 mg/ml of TC and DC (+ their respective epimer if these epimers are contained in the standard).

3.13.3- Epi-OTC, epi-TC and epi-CTC stock standard solution: prepare 3 methanolic solutions containing each 1 mg/ml of epimer.

3.14- Working solutions for analysis of kidney samples:

3.14.1- Two 100µg/ml intermediate solutions are obtained by diluting the two stock solutions (3.13.1- and 3.13.2-) with methanol. These solutions can be stored two weeks at $+4\ ^{0}C$

3.14.2- Working solutions are obtained by diluting each of the two intermediate solutions (3.14.1) with 0.01 M oxalic acid in methanol/water solution (30/70) to obtain concentrations of 0.75; 1.5; 3 and 6µg/ml. These solutions are prepared freshly every day in amber flasks.

3.14.3- Working solutions containing 1µg/ml of each epimer are obtained by diluting each of the stock standard solutions (3.13.3) with 0.01 M oxalic acid in methanol/water solution (30/70). These solutions will be used only for epimers identification and not for quantification. The amount of tetracycline + the corresponding epimer contained in a kidney sample is calculated by comparison with the standard of tetracycline only.

3.15- Working solutions for the analysis of muscle samples

3.15.1- Two 50µg/ml intermediate solutions are obtained by diluting the two stock solutions 3.13.1- and 3.13.2- with methanol. These solutions can be stored two weeks at +4 ºC

3.15.2- Working solutions are obtained by diluting each of the two intermediate solutions (3.15.1) with 0.01 M oxalic acid in methanol/water solution (30/70) to obtain concentrations of 0.125; 0.25; 0.5 and 1µg/ml. These solutions are prepared freshly every day in amber flasks.

3.15.3- Working solutions containing 1µg/ml of each epimer are obtained by diluting each of the stock standard solutions (3.13.3) with 0.01 M oxalic acid in methanol/water solution

(30/70). These solutions will be used only for epimers identification and not for quantification. The amount of tetracycline + the corresponding epimer contained in a kidney sample is calculated by comparison with the standard of tetracycline only.

3.16- Control kidney

3.17- Control muscle

3.18- Spiking solutions

3.18.1- Spiking solutions for kidney samples: Spiking solutions of 6µg/ml are obtained by diluting the two stock solutions with ultra pure water. These solutions can be stored at +4ºC for 24 hours.

3.18.2- Spiking solutions for muscle samples: Two 100µg/ml intermediate solutions are obtained by diluting the two stock solutions (3.13.1 and 3.13.2) with methanol. Spiking solutions of 1µg/ml are obtained by diluting with the hundredth these two intermediate solutions. These solutions can be stored at +4ºC for 24 hours.

3.19- Spiked control samples: The control samples allow calculating the recovery and ensuring the quality of the analysis.

3.19.1- Kidney spiked samples: Prepared fortified kidney samples by adding 500µl of spiking solutions (3.18.1) to 5g of control kidney (3.16) to obtain a spiking level of 600µg/kg. Stir for 30 seconds. The kidney sample is frozen until analysis.

3.19.2- Muscle spiked samples: Prepare fortified muscle samples by adding 500µl of spiking solutions (3.18.2) to 5g of control muscle (3.17) to obtain a spiking level of 100µg/kg. Stir for 30 seconds. The muscle sample is frozen until analysis.

4. Apparatus

4.1- Laboratory equipment

4.1.1- Polypropylene centrifuge tubes, 50 ml capacity, with caps.

4.1.2- Glass tubes, 30 ml capacity.

4.1.3- Polypropylene tubes, 5 ml capacity.

4.1.4- Amber volumetric flasks, 25 ml, 50 ml, 100 ml, 200 ml and 1000 ml.

4.1.5- Graduated glass pipettes, 2 ml, 5 ml, 20 ml and 25 ml.

4.1.6- Automatic pipettes type Gilson P1000.

4.1.7- Blender type moulinette (Moulinex).

4.1.8- Analytic and precision balance model PB302 (Mettler – Toledo).

4.1.9- High precision analytic balance type A120S (Sartorius).

4.1.10- Solvent dispensers (Brandt).

4.1.11- pH-meter (Tacussel).

4.1.12- Electric stirrer type vortex (Bioblock).

4.1.13- Rotary stirrer type Rheax 2 (Heidolph). Vortex mixer was used instead.

4.1.14- Magnetic stirrer type Nuova II (Bioblock).

4.1.15- Cooled centrifuge model GR 4.22 (Jouan).

4.1.16- Solid phase extraction cartridges Bond-Elut C18, 3 cc, 200 mg (Varian).

4.1.17- Solid phase extraction manifold (Supelco), adaptors, needles (Analytichem).Syringe was used in this study for the sample to pass-through the cartridge

4.1.18- Vacuum pump, 0.4 bar, 12 w (Bioblock).

4.1.19- Whatman disposable filter funnels, 25 mm diameter (Whatman, Art. 1922-1800) or 50 ml reservoirs containing these same filters.

4.1.20- Membrance filter holder with filter paper model HVLP 0.45µm (Millipore).

4.1.21- Refrigerated ultra-speed centrifuge model MR 1822 (Jouan)

4.2- High Performance Liquid Chromatography equipment

4.2.1- Series 1050 quaternary gradient pump (Hewlett Packard).

4.2.2- Series 1050 UV-VIS detector (Hewlett Packard).

4.2.3- Vectra 486/66VL computer (Hewlett Packard) and HPLC 2D Chemstation software.

4.2.4- Series 1100 auto sampler (Hewlett Packard).

4.2.5- Analytical column Nucleosile C18,250*4mm,5µm

5. Storage of samples and sampling

Sample must be stored at about -20ºC. They must be thawed just before the analysis and then ground (4.1.7).

6. Procedure

NB: Tetracyclines are sensitive to light. Care must be taken to protect solutions from light during the manipulations.

6.1- Extraction

6.1.1- Weigh out 5 ± 0.1 g of ground kidney or muscle into a centrifuge tube (4.1.1).

6.1.2- Add 25 ml McIlvain buffer/ETDA solution (3.10) and stir for about 30 s (4.1.12).

6.1.3- Stir for 15 min at 100 rpm with the rotary stirrer (4.1.13).

6.1.4- Centrifuge 10 min at 4000 g about 4ºC. Do not leave the samples for a long time in this state because of problems of stability.

6.2- Proteins precipitation

6.2.1- Transfer the supernatant in a glass tube (4.1.2), place this tube in a beaker on the magnetic stirrer (4.1.14).

6.2.2- Add slowly 2.5 ml of 1 g/ml trichloroacetic acid solution (3.3) with constant stirring. Then stir more rapidly for a further 1 min. Remove the magnetic stirrer.

6.2.3- Centrifuge 5 min at about 3000 g.

6.3- Cleanup

6.3.1- Activate the cartridge Bond Elute with 1 ml methanol, 1 ml ultra pure water and 1 ml McIlvain buffer. (3.9).

6.3.2- Connect a filter funnel or a reservoir containing a filter (4.1.19) to the cartridge.

6.3.3- Transfer the sample solution into the funnel and pull it through the filter with the vacuum pump (4.1.18) at a flow rate of no more than 2 drops/s. Do not allow the cartridge to dry at this step.

6.3.4- Flush the cartridge with 1 ml ultra pure water.

6.3.5- Dry the cartridge for 5 min using the vacuum pump.

6.3.6- Remove the filter and elute slowly with 1 ml 0.01 M oxalic acid in methanol (3.5) and next with 1 ml ultra pure water into a polypropylene tube (4.1.3).

6.3.7- The samples are centrifuged 3 min at 20,000 g at about 4^{0}C before injection of a 100-µl volume into the chromatographic system.

6.4- Chromatographic conditions

6.4.1- Gradient mobile phase

Time	Acetonitrile, %	0.01 M oxalic acid, %
0 min	13	87
15 min	36	64
Post-time: 5 min.		

6.4.2- Flow rate: 0.8 ml/min.

6.4.3- UV detector wavelength: 355 nm.

N.B.: in case of chlortetracycline analysis, the wavelength can be set at 375 nm, which is the more adjusted wavelength for chlortetracycline detection.

6.4.4- Retention times:

Oxytetracycline: 10.5 min

Tetracycline: 12.5 min

Doxycycline: 18.9 min

7- Calculation of results

The following calculations can be executed directly by the HPLC 2D Chemstation software.

7.1- Derive the calibration curve from the results obtained with the working standards solutions. Peaks corresponding to the tetracyclines and to their respective epimer have to be taken into account if possible. Then, determine the curve equation:

$y = ax + b$

y = peak area (TC + epimer)

x= concentration (ng/ml)

a = slope

b = intercept

7.2- Calculation of the recovery:

This result is obtained from the spiked control sample.

Determine the control sample final concentration (Cf) using the curve equation (7.1) as:

$$Cf = \frac{Yf - b}{a}$$

Cf = final concentration of the injected extract

Yf = tetracycline peak area + epi-tetracycline peak area

a = slope

b = intercept

Calculate the recovery as:

$$R = \frac{Cf}{F.\ Ct}$$

R = recovery

Cf = final concentration of the injected extract determined above

Ct = true concentration or spiking concentration (600µg/kg = MRL)

F = concentration factor (2.5 in this case).

Check the quality of the analysis: this last is validated if the calculated recovery is in accordance with the limits establishing during the method validation:

$Rm - 3.SD \leq R \leq Rm + 3.SD$

R = recovery

Rm = mean recovery determined during validation

SD = standards deviation of the mean recovery

Calculate the concentration of tetracycline + epi-tetracycline present in the sample to be analyzed (Ca) using the calibration curve and taking account the calculated recovery:

$$Ca = \frac{Cf}{F} \times \frac{1}{R}$$

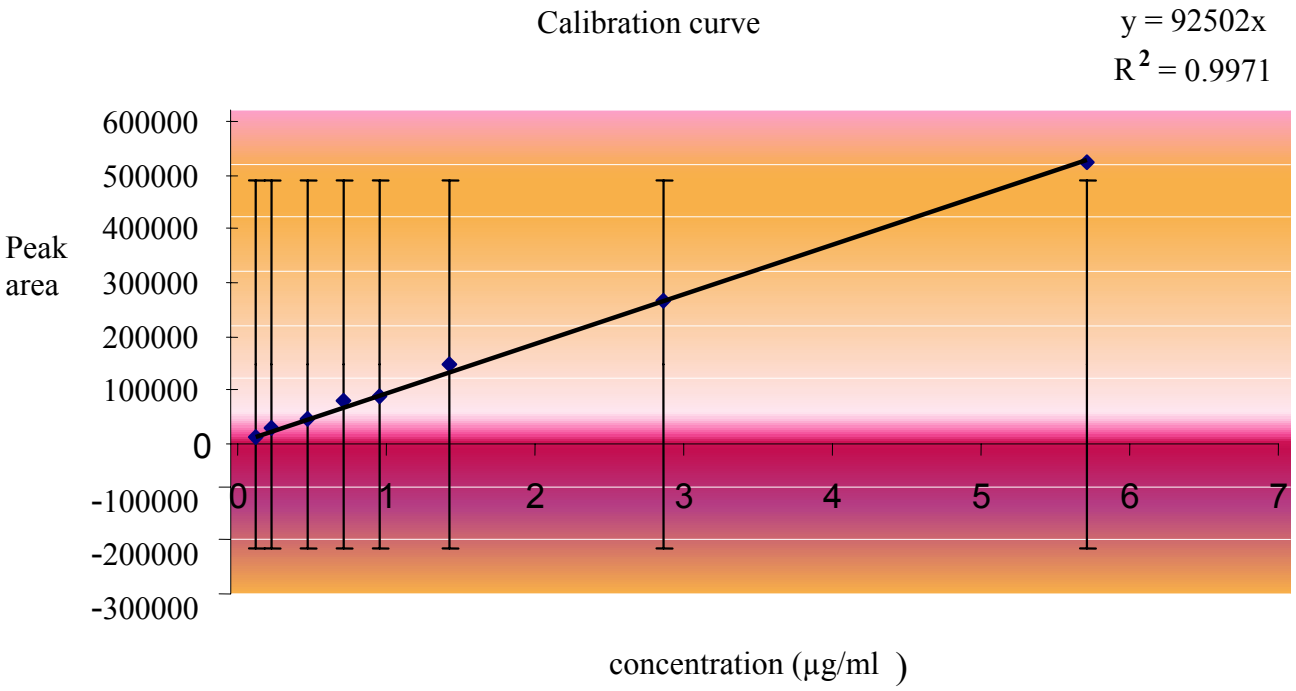
Ca = concentration of tetracycline + epi-tetracycline present in the sample to be analyzed.

Cf = final concentration of the injected extract.

R = recovery

F = concentration factor (2.5 in this case).

Annex 2. Derived calibration curve and its equation from the results obtained with the working standard solutions.



Annex 3. Principles and Practice of thin layer chromatography (Sherma and Fried,1996)

1. Scope and field of application

Thin layer chromatography (TLC) and high performance thin layer chromatography (HPTLC) now also called planar chromatography; are, like all chromatographic techniques, based on a multistage distribution process. This process involves: a suitable adsorbent (the stationary phase), solvents or solvent mixtures (the mobile phase or eluent), and the sample molecules. For thin layer chromatography the adsorbent is coated as a thin layer onto a suitable support (e.g. glass plate, polyester or aluminium sheet). On this layer the substance mixture is separated by elution with a suitable solvent.

2. Sample preparation

For a chromatographic separation the sample must meet several requirements to obtain good results. Several steps for sample pretreatment may be necessary These include sampling, mechanical crushing of a sample, extraction steps, filtration and sometimes enrichment of interesting components or clean-up, i.e. removal of undesired impurities. The examples of the beginner's set do not require complicated procedures. The advanced sets require the user to carry out some additional steps for preparing a sample. Thorough preparation of samples is an important prerequisite for the success of a thin layer chromatographic separation.

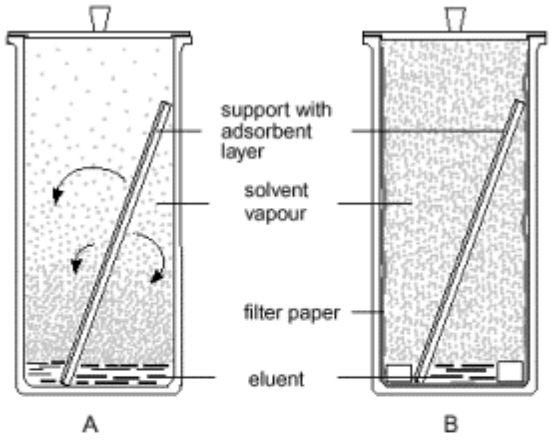
3. Sample application

The aim of a chromatographic separation determines how the sample should be applied to the TLC plate or sheet. The most frequent technique still is application with a glass capillary as spot or short streak. Application as streak will yield better results especially for instrumental quantification. Large spots will cause poor separations since during chromatography they will become even larger and more diffuse. Draw a straight line 2 cm from the bottom of the plate The mixture to be separated and the reference solution are applied as spots by means of glass capillaries 1.5 cm apart from each other. Place the capillary on the layer vertically and carefully, vertically so that the capillary empties itself and carefully to avoid damage to the layer. Damaged layers result in unevenly formed spots. To keep spots as small and compact as possible, it is

advisable to apply a solution in several portions with intermediate drying (blow with cold or hot air).

4. Developing a chromatogram (separation techniques)

The most frequently used separation technique is ascending TLC in the customary trough chamber (standard method, linear development). Usually it is applied as single development. However, multiple development, with or without change of eluent (step technique) can improve separation results. Saturation of the chamber atmosphere with eluent vapor is necessary.



Developing chambers and saturation
A) chamber with normal saturation. The arrows stand for the evaporation of the eluent from the layer and the dots symbolise the vapour density.
B) chamber lined with filter paper, saturated with eluent

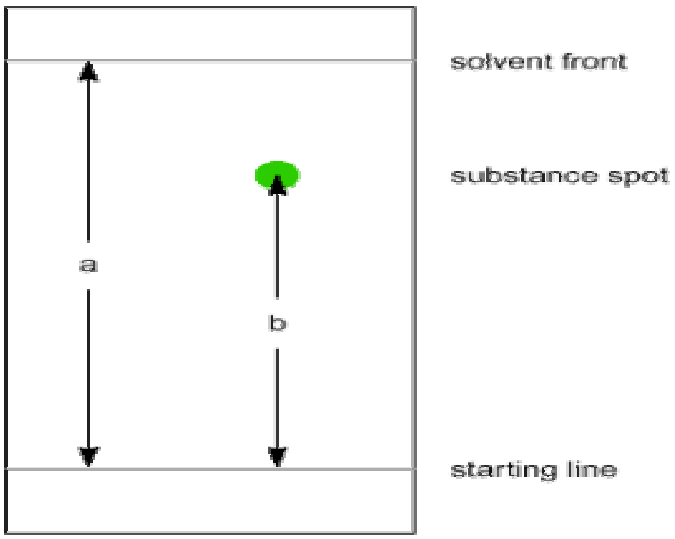
5. Evaluation of a thin layer chromatogram

The evaluation depends on the purpose of a chromatographic analysis. For qualitative determination often localization of substances is sufficient. This can be easily achieved by parallel runs with reference substances. A parameter often used for qualitative evaluation is the Rf value (retention factor). The Rf value is defined as follows:

$$R_f = \frac{\text{distance starting line – middle of spot}}{\text{distance starting line – solvent front}} = \frac{b}{a}$$

The R f values are between 0 and 1, best between 0.1 and 0.8. If reproducible R f values are to be obtained it is, however, essential that several parameters such as chamber saturation, constant

composition of solvent mixtures, constant temperature etc. are strictly controlled. A quantitative evaluation is possible by suitable calibration measurements. For this purpose either the area of a substance spot is measured or a photometric evaluation is performed directly on the layer.

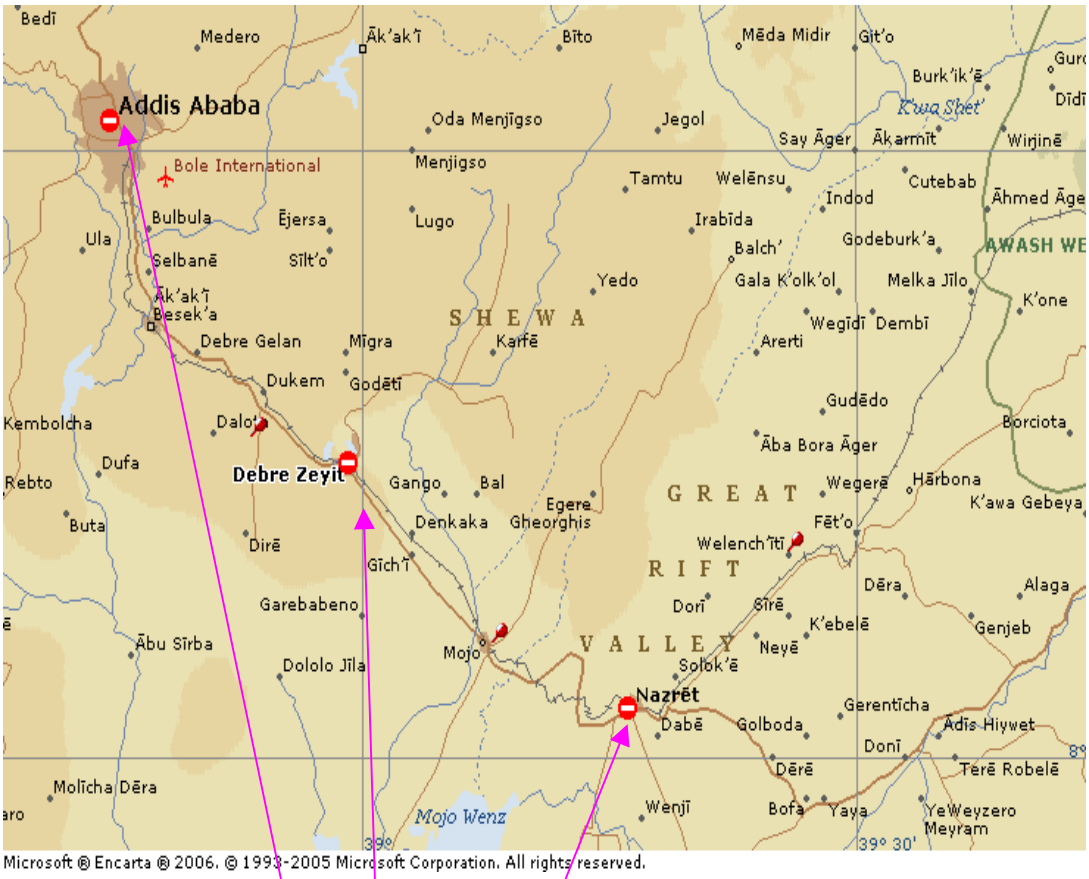


The latter procedure, however, requires a higher instrumental expense. Qualitative detection is generally made directly on the TLC plate via the characteristic Rf values of substances and color of the spot.

5. Visualization of separated substances

Only in very few cases the sample is a dye which can be seen with the naked eye. Much more often for unspecific visualization substances can be viewed under UV light, since many substances show a UV absorption. If a fluorescent indicator is added to the layer, all substances absorbing in the respective region of wave length cause a quenching of the fluorescence, i.e. they appear as dark spots on the fluorescent layer. Customary fluorescent indicators are excited at 254 nm or (less frequently) at 366 nm with a mercury lamp. For a number of compounds their native fluorescence can be used for visualization, which is excited by UV light (mostly long-wave UV) (e.g. yellow in case of tetracycline). This allows not only determination of the R f value, but often enables a further qualitative assignment. If these methods do not allow localization or characterization of substances, especially post-chromatographic detection methods can be applied, chemical reactions on the plate. Quite unspecific reactions are iodine adsorption and the charring technique (spraying with sulphuric acid and heat treatment).

Annex 4. Map of study areas



Source: Microsoft@Encarta, 2006

9. CURRICULUM VITAE

Personal data

Name	Addisalem Hunde
Date of Birth	May 14, 1981
Place of birth	Arsi Zone, Assela
Sex	Male
Nationality	Ethiopian
Marital status	Unmarried
Children	No
Religion	Orthodox Tewahido
Language Proficiency	Amharic,Oromifa and English
Contact	addiyehunde@yahoo.com

Educational background

1981-1985 E.C.	Sagure Elementary School
1986-.1991 E.C.	Chilalo Terara Senior Secondary School (Assela).
1992 E.C.	Addis Ababa University, Faculty of Science, Addis Ababa (Fresh man courses).
1993-1997 E.C.	Addis Ababa University, Faculty of Veterinary Medicine, Debre Zeit (Courses of general Veterinary medicine).
1998-1999 E.C.	Addis Ababa University, Faculty of Veterinary Medicine, Debre Zeit (Master of Veterinary Science in Veterinary Pubic Health).

Research paper

- Health and Welfare problems of working Equine in Southern Nations and Nationalities Regional State (SNNR)of Ethiopia. DVM Thesis (2005) FVM, AAU, (Unpublished).

Other papers

- Rabies : Diagnosis, Treatment, Prevention and Control. Seminar paper (2004).
- Veterinary Drug Residues in Food of Animal Origin and their Public Health importance Seminar paper (2006).

Additional trainings and certificates

- Computer literacy: Basic computer application software courses, June/2003-December 15/2004. (Diploma).

Work experience

As externship student from September, 2004-June, 2005 in Awassa Agricultural college, abattoir work, laboratory, clinical work activities and application of Chromatographic techniques in the analysis of Veterinary Drugs in the years 2006 - 2007 at the laboratory of Drug Administration and Quality Control of Ethiopia

10. SIGNED DECLARATION SHEET

I, the undersigned, declare that the thesis is my original work and has not been presented for a degree in any other university and that all sources of materials used for the thesis have been duly acknowledged.

Name: Addisalem Hunde

Signature.....
Date of submission.....

This thesis has been submitted for the examination with my approval as advisor.

Dr. Bayleyegn Molla	Signature	_____
(Associate professor)		
Ato Wondie Alemu	Signature	_____