LEVELS OF ANTIBIOTIC RESIDUES IN COW'S MILK SOLD IN COMMERCIAL CENTERS OF KAMPALA CITY

BY

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DECLARATION

I, Eva Namutebi (15/U/14522/GMFT/PE), declare that the work contained in this report is original and has never been submitted to any institution of higher learning for a similar or other award.

Signed.....Namutebi..... Date.....02/11/2018.....

APPROVAL

This is to certify that the work presented herein this report is the student's own, done by her under our guidance and is now ready for submission to the Board of Examiners of Kyambogo University.

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DEDICATION

Dedicated to my dear husband Dr. Nyende David Livingstone and children Nangobi Gladys Davida, Byogero Donah Beatrice and Kakaire Dawson David.

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Glory to the Almighty God.

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LIST OF ABBREVIATIONS AND ACRONYMS

CAC	Codex Alimentarius Commission
CRS	Certified Reference Standards
EU	European Union
FAO	Food and Agriculture Organization
GDP	Gross Domestic Product
KYU	Kyambogo University
LC	Liquid Chromatography
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
MRL	Maximum residue limit
MS	Mass spectrometry
ND	Not detected
NDA	National drug authority
Ppm	parts per million
rpm	Revolution per minute
SPE	Solid Phase Extraction
TC	Tetracycline
USFDA	United States Food and Drug Administration
µg/kg	Microgram per kilogram
µg/l	microgram per liter
WHO	World Health Organization

ABSTRACT

Veterinary drug residues in milk represent a health risk for the consumer especially young children. Prolonged exposure to antimicrobial residues in food and milk may lead to antimicrobial resistance and other health problems. The objective of this study was to evaluate the types of antibiotic residues and their levels in milk, and the health risks posed to milk consumers in Kampala City. A total of one hundred twenty five samples (25 processed and 100 raw) were randomly obtained from different commercial points in Kampala. The milk was analyzed for quinolone, tetracycline, aminoglycoside, sulfonamide, amphenicol and ß-lactam groups of antibiotics using liquid chromatography tandem mass spectrometry (LC/MS/MS). Residue levels of antibiotics in processed milk were in the range of; not detected to 0.0472 µg/l, not detected to 0.0056 µg/l, 1.2231 to 32.3927 µg/l, 1.8836 to 25.4283 µg/l, 0.0001 to 0.0006 µg/l, 0.0143 to 0.113 µg/l for quinolone, tetracycline, aminoglycoside, sulfonamide, amphenicol and ß-lactam, respectively. The corresponding levels in raw milk ranged from not detected to 0.0309 µg/l, not detected to 0.0309 µg/l, 1.1795 to 31.351 µg/l, 1.6698 to 38.2626 µg/l, not detected to 0.0004 µg/l and not detected to 0.5109 µg/l. Levels of aminoglycoside were generally high compared to other groups of antibiotics in milk. However, drug residue levels were in all cases below the maximum residue limit (MRL) according to Food and Agriculture Organization (FAO) and United States Food and Drug Administration (USFDA). Based on the observed levels of antibiotics, processed and raw milk in Kampala is safe for human consumption. Nevertheless, regular monitoring of antibiotic residues in milk is recommended.

CHAPTER 1: INTRODUCTION

1.1 Background

Agriculture is the most important sector of Uganda's economy (Tijjani & Yetisemiyen, 2015). The dairy sector is one of the agricultural sectors in Uganda that has enjoyed sustained growth to-date (Ndambi et al., 2008; Mwebaze & Kjaer, 2013). It contributes about 50 % of total output from the livestock sector, 20 % of the food processing industry and 4.3 % of the National Gross Domestic Product (GDP) (Ndambi et al., 2008). Uganda's milk output was *ca.* 1.7 billion litres in 2011 (Tijjani & Yetisemiyen, 2015). Milk production is reported to be growing very fast, and Uganda is among the group of African countries with the highest growth rates (Ndambi et al., 2008). Similarly, consumption is on the increase, and like in most other developing countries, milk consumption is higher in the cities than in rural areas. Kampala, the Capital City of Uganda, is the major consumption centre (Ndambi et al., 2008).

In order to meet the increasing demands for milk in Uganda, dairy production has become intensive (Atuhaire et al., 2014). Milk is a source of protein of high quality, fat and sugar (lactose). It is also a source of vitamins, mineral elements including phosphorous, calcium, magnesium, potassium and sodium (Mourad et al., 2014; Haug et al., 2007). Safe milk and milk products are a rich and convenient source of nutrients (Muhib et al., 2016).

Drug residues of veterinary origin including growth hormones, anthelminthic drugs and antibiotics used in animal production may occasionally be found in milk and other animal products such as meat (Khaniki, 2007). The occurrence of antibiotic residues in milk is a matter of public health concern because it can cause allergic reactions and antimicrobial resistance

especially in children (Ibraimi et al., 2013; Bilandžić et al., 2011). Antibiotics are antimicrobial substances that are produced either naturally by microorganisms or synthetically through laboratory procedures and have the ability to inhibit the growth of, or entirely destroy microorganisms (Darwish et al., 2013). They are widely used in livestock production for many purposes including disease treatment and prevention, and for feed efficiency as growth promoters (Layada, Benouareth, Coucke, & Andjelkovic, 2016; Tollenfson & Miller, 2000). However, antibiotics have also been implicated in causing permanent gene mutation, liver poisoning, immuno-pathological effects, hypersensitivity, carcinogenicity, mutagenicity, nephropathy and disruption of intestinal normal flora (Boursi et al., 2015; Darwish et al., 2013). In addition, they affect the technological properties of milk by inhibiting the growth of starter cultures used in the manufacture of cultured dairy products such as yoghurt and cheese (Orwa et al., 2017; Aalipour et al., 2015).

In Uganda, information on antibiotic residues in milk, and the health risk that they may pose to the consumer is limited. Antibiotics used in livestock production are not well regulated in many developing countries (Mainda et al., 2015). Many of the antibiotics also serve as essential medicines for humans (Annan-Prah et al., 2012). The use of antibiotics in dairy animals has the potential to generate residues in milk. Regular consumption of milk contaminated with antibiotic residues increases the risk of antimicrobial resistance among children, HIV/AIDS patients and hospitalized surgical patients (Seni et al., 2013). In vitro resistance of *Streptococcus pneumonia* to co-trimoxazole and penicillin treatment has been observed (Rutebemberwa et al., 2015). Pneumonia is the second major cause of death in children < 5 years of age in Uganda

(Rutebemberwa et al., 2015). There is limited information on the levels of antibiotic residues in both processed and unprocessed milk in Kampala.

1.2 Problem statement

In Uganda, antibiotics are frequently used in animal production. These antibiotics may find their way in animal products such as milk. Antibiotic residues are associated with loss of beneficial anaerobic organisms and increase in potentially pathogenic microbes in intestinal microbiota, leading to decreased conversion of health-promoting compounds such as phytochemicals to biologically active compounds that could play an inhibitory role in carcinogenesis. Antibiotics may also increase the bacterial production of toxins and decrease the number of bacteria which prevent tumorigenesis (Cao, et al., 2016; Kilkkinen, et al., 2008; Velicer, et al., 2004). Thirty six percent and seventy percent of the Ugandan population consume milk daily and at least once in a week respectively (Tijjani & Yetisemiyen, 2015). Of the total milk consumed in Uganda, about 80% is unprocessed and obtained through informal market segments with no proper regulatory mechanisms (Mwebaze & Kjaer, 2013).

1.3 Justification of the study

Understanding of the quality and safety of milk sold in the commercial centers of Kampala City has ultimate benefits to the consumers of milk and other dairy products. In addition equal benefits are obtained by the producers of milk (farmers) and dairy industry is assured of good milk that can be safely processed to other products. It was therefore envisaged that this study will add information to the chemical safety and quality of milk in Uganda and provide baseline data for further investigation on milk safety. The obtained information would also assist milk

producers, Regulatory bodies and consumers to contribute to the formulation of control strategies on the use of antibiotics as veterinary drugs in treating and preventing various cattle diseases. It is suspected that milk products contain antibiotic residues. This study was therefore conducted with the view of generating baseline data that could be used to guide policy formulation, and also for purposes of consumer protection.

1.4 Hypotheses

1. The milk sold in commercial centers in Kampala city contains different types of antibiotic residues.
2. Milk consumed in commercial centers of Kampala city poses a health risk because of elevated levels of antibiotic residues.

1.5 Objectives of the study

1.5.1 Overall objective

The general objective of the study was to assess the nature of antibiotic residues and their levels in milk, and the health risks posed to milk consumers in Kampala city.

1.5.2 Specific objectives

1. To determine the different types of antibiotic residues in raw and processed cow's milk in commercial centers of Kampala city.
2. To quantify the levels of the antibiotic residues in raw and processed cow's milk in commercial centers of Kampala city.

CHAPTER 2: LITERATURE REVIEW

2.1 Milk production

Milk plays a very important role in improving people's nutritional status and in income generation in Uganda (Ndambi et al., 2008). Global milk production is dominated by milk from 5 animal species: dairy cattle, buffalos, goats, sheep, and camels. According to FAOSTAT, the total world milk production in 2009 was 696.6 million kg^3 of which 83.3% was cow's milk, 13% buffalo milk, 2.2% goat milk, 1.3% sheep milk, and 0.2% camel milk (Barłowska et al., 2011). Although goats, camels and buffaloes are listed among the dairy animals, cattle are the major source of milk in Uganda (Tijjani & Yetisemiyen, 2015). The major cow milk producers worldwide are the European Union at 148.1 million kg^3, the United States of America at 85.9 million kg^3, India at 45.1 million kg^3 and Russia at 32.3 million kg^3 (Barłowska et al., 2011). The dairy sector in Uganda has grown rapidly at about 7 percent per annum over the last 30 years (Mwebaze & Kjaer, 2013). The evolution of milk production in Uganda for the period between 1996 and 2016 (Figure 1), milk production increased five times from 500, 000 liters in 1998 to 2, 500, 000, 000 liters in 2014. Milk production in Uganda is dominated by five regions including South-western, Central, Eastern, Northern and Karamoja in descending order (Ekou, 2014; Mwebaze & Kjaer, 2013; Sikawa & Mugisha, 2011)

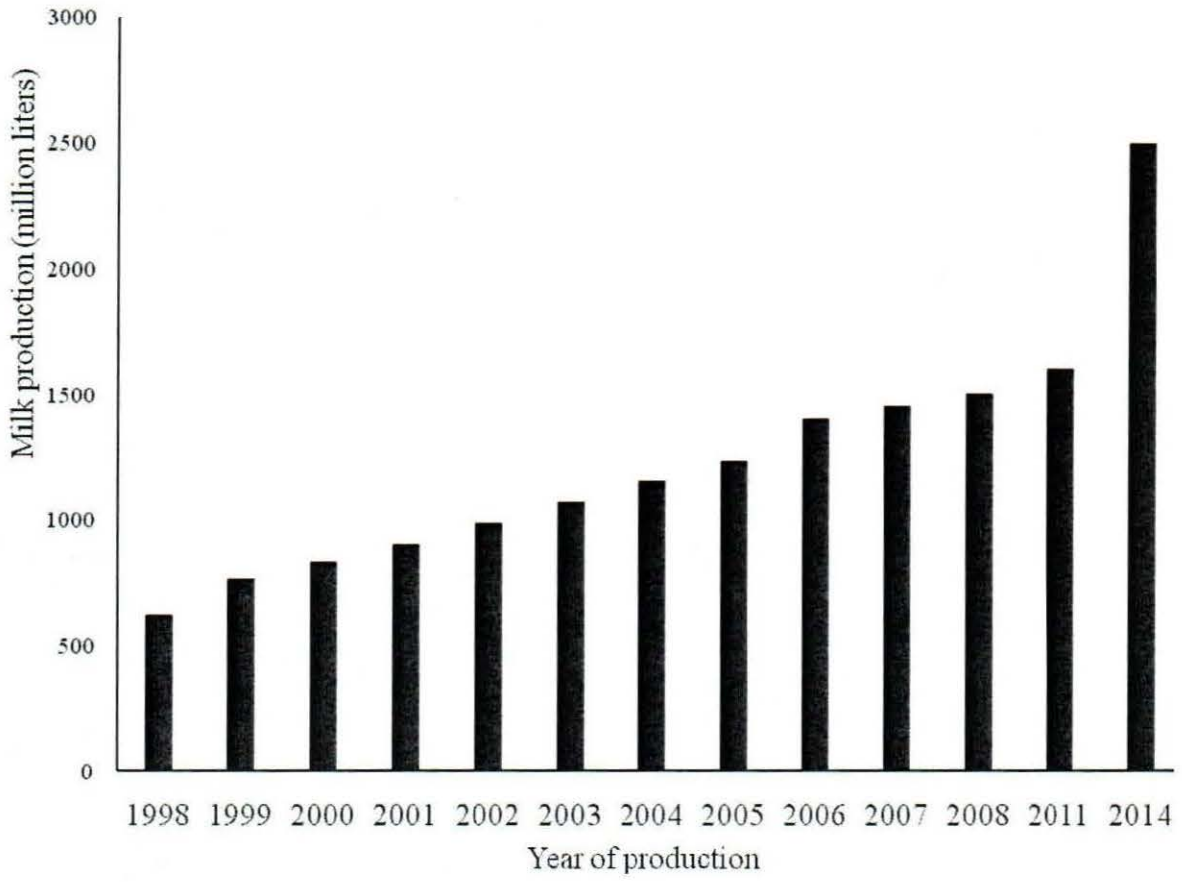

Figure 1: Milk production in Uganda between 1998 and 2014.

(Source: Wozemba & Nsanja, 2008; Mwebaze & Kjaer, 2013; J.Ekou, 2014))

2.2 World milk consumption trends

The per capita consumption of milk and milk products in developing countries has grown tremendously due to growing incomes, population growth, urbanization and change in diets (Kasirye, 2015). Milk consumption in Uganda has more than doubled from 20 to 44 litres per person per year (Mwebaze & Kjaer, 2013). According to FAO, Uganda's per capita consumption of milk is rated as medium on the global scale i.e., between 30 and 150 liters (Table 1).

Table 1: Global per capita milk consumption according to FAOSTAT

Rate	kg/capita/year	Country/Region
High	> 150	Argentina, America, Australia, Coasta Rica, Europe Israel, Kyrgyzstan, North America & Pakistan
Medium	30 – 150	Uganda, India, Islamic Republic of Iran, Japan, Kenya, Mexico Mongolia, New Zealand, North & Southern Africa, Southern Africa, most of Latin America & Caribbean
Low	< 30	Viet Nam, Senegal, most of Central Africa & most of East & Southeast Asia

Source: Kasirye (2015)

2.3 Market segments

There are two milk market segments, namely the formal and informal segments. Informal segment refers to the traditional way of marketing in which raw milk is procured from farmers and sold directly to consumers without prior processing and packaging (Sikawa & Mugisha, 2011). The formal milk marketing channel operates an organized system of milk collection using well established bulking centers with coolers and transport infrastructure (Nkwasibwe et al. 2015). Only 20% of the total milk production in Uganda is processed, meaning that the 80% is marketed through informal means (Balirwa et al. 2016; Tijjani & Yetisemiyen, 2015; Nkwasibweet al.2015; Balikowa, 2011).

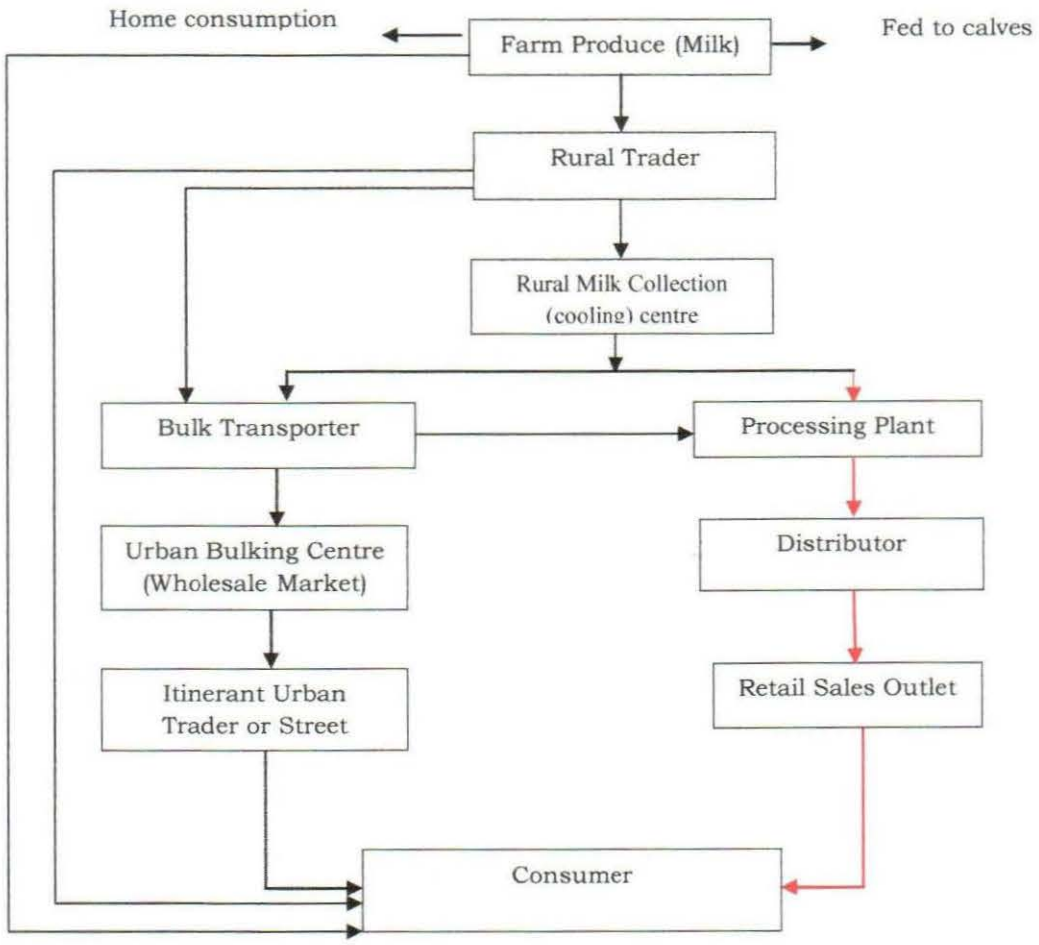

Figure 2: Flow of milk through the Formal and Informal market segments

Formal Channel

Informal Channel

(Source: Balikowa, 2011)

2.4 Nutrition composition of milk

The chemical composition of milk can be Influenced by several factors such as animal species and genetics, environmental conditions, lactation stage, and animal nutritional status (Pereira, 2014). When comparing cow milk with sheep and goat, as well as with human milk, some differences can be pointed out. Sheep milk can be distinguished by its higher protein and fat

content while goat milk has higher amounts of A, B1 and B12 vitamins as well as calcium and phosphorus content when compared to cow and sheep milk (Pereira, 2014). The nutritional composition of sheep, goat, cow and human milk is presented in Table 2. In general milk is an important source of protein in the human diet, supplying approximately 32 g protein/L.

Table 2: Composition of milk from various animal species (per kg)

Component	Sheep	Goat	Cow	Human
Energy (Kcal)	690 – 2530	670 – 2530	674 – 2750	677 – 680
Lactose (g)	47 – 49	41 – 51	47 – 50	67 – 69
Protein (g)	52 – 62	34 – 36	32 – 36	12 – 14.2
Fat (g)	64 – 79	38 – 42	36 – 41.4	36.4 – 40
Ash (g)	8.5 – 9.2	7.7 – 8.6	7.0 – 7.6	2.2
Calcium (mg)	120 – 122	100 – 134	120 – 122	33
Phosphorus (mg)	119	121	119	43

Source: Potočnik, et al., (2011); Guetouache, et al., (2014)

2.5 Antibiotic use in cattle

In order to meet the increasing demands for milk by the ever increasing human population, dairy production has become increasingly intensive (Atuhaire et al., 2014). Intensive dairy farming demands increased use of antibiotics; they are needed to meet the challenges of providing adequate amounts of food for the growing population. They improve the rate of weight gain and feed efficiency, and are used to prevent and treat diseases in humans and animals (Layada et al., 2016; El Atabani et al., 2014; Singh et al., 2014; Moyane et al., 2013). However, the benefit of

improved production is associated with the bioaccumulation of antibiotic residues in animal products. Antibiotic residues are pharmacologically active substances whether active ingredients, recipients or degradation products and their metabolites which remain in foodstuffs obtained from animals to which the antibiotic in question has been administered (Mensah et al., 2014).

Improper use of antibiotics, inadequate knowledge of the necessary withdrawal time and individual characteristics like health of the animal, amount and type of applied antibiotics, quantity of milk production, and method of antibiotics application can easily make the antibiotics or their derivatives appear in milk. Parenterally applied antibiotics are excreted much faster through milk, while with intramamary application, residues are found in higher concentrations and last for longer periods (Khaskheli et al., 2008; Fejzić et al., 2014 ; Aalipour et al., 2015). The antibiotics considered in the study appeared on the NDA register of the approved drugs for veterinary use in Uganda as of April 2017 (NDA, 2017).

2.6 Classification of antibiotics

There are several ways of classifying antibiotics but the most common classification schemes are based on their molecular structures, mode of action and spectrum of activity (Adzitey, 2015 ; Etebu & Arikekpar, 2016). Others include route of administration i.e., injectable, oral or topical. Antibiotics within the same structural class generally show similar pattern of effectiveness and potential toxic effects (Etebu & Arikekpar, 2016). However, variations in the properties of antimicrobials within a class often arise due to different side chains in the molecule. Based on their chemical structure, antibiotics are categorized into beta-lactams, macrolides, tetracyclines,

quinolones, aminoglycosides, lincosamides and sulphonamides as the major classes (Orwa, et al., 2017).

2.6.1 Beta-lactams

Beta-lactams are the oldest and the most commonly used antibiotics. They are characterized by the presence of the beta-lactam ring which is chemically highly reactive. Beta-lactams are used especially to fight mastitis, a disease that causes considerable economic losses in the dairy industry (Ibraimi et al., 2013). Examples of beta-lactams include penicillin and cephalosporin antibiotics. In penicillins, the beta-lactam ring is fused with a five-membered thiazolididine ring while in the - cephalosporins, the ring is fused with a six-membered dihydrothiazine ring (Kebede et al., 2014). They are bactericidal and act by disrupting peptidoglycan synthesis in actively multiplying bacteria (Wivagg et al., 2014)

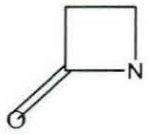

Figure 3: Beta-lactam ring

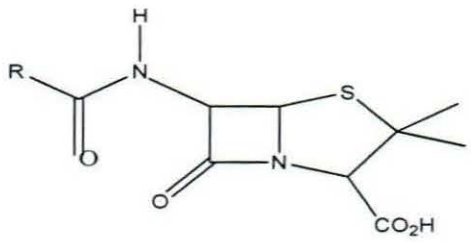

Figure 4: Penicillin nucleus

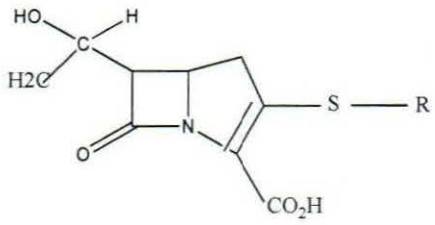

Figure 5: Carpanem nucleus

2.6.2 Macrolides

The macrolide structure is characterized by 12–16 lactone rings. Macrolide drugs are complex mixtures of closely related antibiotics that differ from one another in the chemical substitutions on the various carbon atoms in the structure. They also differ in the types of amino and neutral sugars present in the molecule. An example of macrolides is tylosin, which is used globally as a broad spectrum antibiotic against a wide range of Gram-positive and Gram-negative aerobic and anaerobic bacteria (Adzitey, 2015; Bilandžić et al., 2011). Their activity is based on their ability to inhibit protein synthesis by binding on to the 50*S* subunit of the ribosome, resulting in inhibition of translocation reactions, protein synthesis, and consequently bacteria cell growth.

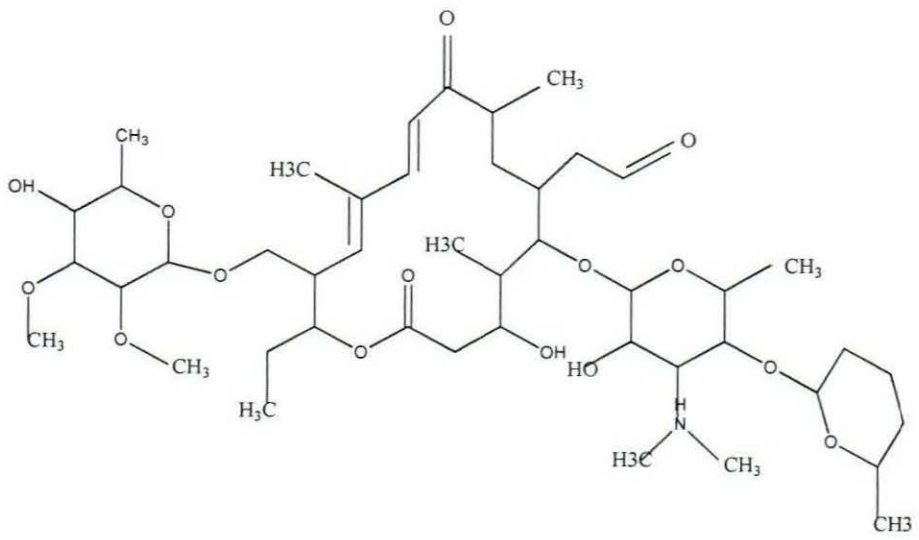

Figure 6: Macrolide structure

2.6.3 Tetracyclines

Tetracyclines have a hydronaphthacene nucleus containing four fused rings. They are broad-spectrum antibiotics that inhibit protein synthesis. They act by blocking binding of the aminoacyl-tRNA to the acceptor site on the mRNA-ribosomal complex and prevent the formation of the initiation complex required for polypeptide chain synthesis (Hussain et al., 2015; Adzitey, 2015; Bilandžić et al., 2011). Four types of tetracycline antibiotics are routinely used in animal husbandry. They include; oxytetracycline (OXT), tetracycline (TET), chlortetracycline (CHT) and doxycycline (DOT) (Aalipour et al., 2015). Tetracycline is partially excreted through milk. Its concentration in milk is approximately 70 % of the concentration within the serum (Aalipour et al., 2015).

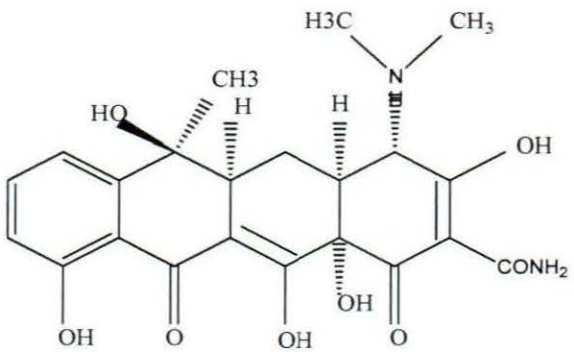

Figure 7: Hydronaphthacene nucleus

2.6.4 Quinolones (fluoroquinolones)

The World Health Organisation (WHO) and World Organization for Animal Health (OIE) define quinolones as critically important antibiotics for human and animal health, respectively (Pavlína et al., 2011). The most critical changes to the quinolone skeleton were the introduction of fluorine at position C-6 and a major ring substituent (piperazine or methyl-piperazine) at C-7. Because of the inclusion of the fluorine, quinolones are often termed fluoroquinolones. Fluoroquinolones are active against some Gram-negative bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa,* and *Neisseria gonorrhoea*, and also against Gram-positive bacteria such as *Staphylococcus aureus* and *Streptococcus pneumoniae*. The toxicity and side effects of quinolones are well established in animals and humans. They show damage to the juvenile joint, the kidney, the eye, and the central nervous system (Buket et al., 2013). Quinolones include cinoxacin, norfloxacin, ofloxacin, ciprofloxacin and temafloxacin (Etebu & Arikekpar, 2016). Ciprofloxacin is one of the most commonly prescribed antibacterial drugs used to treat a variety of Gram-negative and, to a lesser extent, Gram-positive infections (Bilandžić et al., 2011; Aldred et al., 2014; Ramatla et al., 2017).

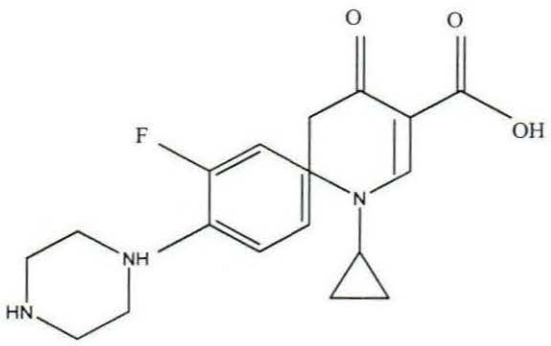

Figure 8: Quinolone Structure

2.6.5 Aminoglycosides

Aminoglycosides are multifunctional hydrophilic sugars that possess several amino and hydroxyl functionalities and have a broad spectrum of antibacterial activity (Kotra et al., 2009). They are effective against aerobic Gram-negative rods (*Escherichia, Pseudomonas, Shigella* and *Salmonella*) and certain Gram-positive bacteria (Bilandžić et al., 2011). Aminoglycosides inhibit protein synthesis and or alter the integrity of bacterial cell membranes. Their activity is dose-dependent moreover they have a significant post-antibiotic effect. The most commonly used aminoglycoside in animal husbandry is gentamicin. Neomycin, streptomycin and dihydrostreptomycin are also used to a lesser extent (Etebu & Arikekpar, 2016).

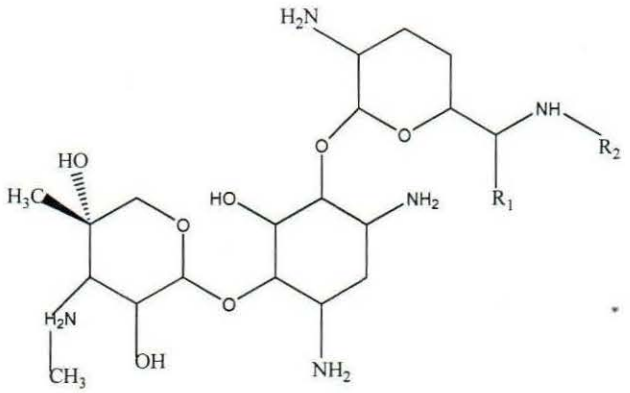

Figure 9: Aminoglycosides structure

2.6.6 Sulfonamides

Sulfonamides are represented by general structure shown in Figure 10. The side chain R may be alkyl, aryl or hetero aryl groups and R1or R2 may also be hydrogen, alkyl, and aryl or hetero aryl groups. The sulfonamides or sulfa drugs competitively inhibit folic acid synthesis in micro-organisms and subsequently inhibit their multiplication but do not kill them (Pareek et al., 2013). The most commonly used sulfonamides include, sulfadiazine, sulfamethazine (sulfadimidine), sulfathiazole, sulfamethoxazole, sulfadoxine and sulfadimethoxine. Sulphonamides inhibit Gram-positive bacteria such as *Nocardia, E. coli, Klebsiella, Salmonella* and *Shigella* and Gram-negative bacteria such as *Enterobacter* and *Chlamydia trachomatis* (Etebu & Arikekpar, 2016). Potentiated sulfonamides, in which a sulfonamide and an antibacterial diaminopyrimidine such as trimethoprim are combine (Ramatla et al., 2017) are more efficacious than non-potentiated molecules (Kebede et al., 2014).

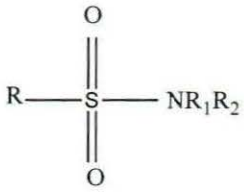

Figure 10: Sulfonamide structure

2.6.7 Phenicols

The phenicol class includes chloramphenicol, florfenicol and thiamphenicol (Adzitey 2015; Bilandžić et al., 2011). They have a time-dependent bacteriostatic effect for most Gram-positive bacteria and many aerobic Gram-negative bacteria.

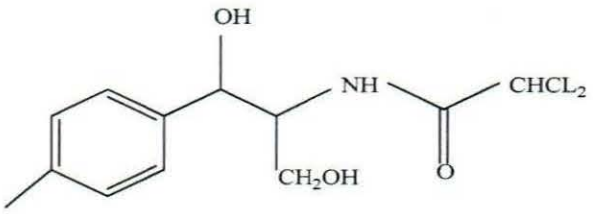

Figure 11: Phenicol Structure

2.7 Monitoring of antibiotic residues in milk

The reasons for monitoring veterinary drug residues in foodstuffs and foods of animal origin include the ethical ones (preventing undesired exposition of healthy consumers to therapeutic doses of drugs in food), hygienic (protection against possible harmful effects of the residues on the consumer's health), technological (preventing the disruption of the fermentation processes), and ecological (Navrátilová, 2008). Different methods including microbial inhibitor methods and rapid specific assays have been used for extensive monitoring of antimicrobial agents. Screening methods for establishing antimicrobial residues in milk include receptor binding and enzymatic colorimetric assays; enzymatic colorimetric assays; receptor binding assays and microbial receptor assays (Navrátilová, 2008). However nowadays, liquid chromatography with tandem mass spectrometry (LC-MS/MS) is the most commonly used tool for detecting a large number of veterinary drug residues in food. It is a robust method, highly selective and very sensitive as well (Layada et al., 2016; Jayalakshmi et al., 2017).

2.8 Maximum residue limits (MRL) for antibiotics in milk

To ensure consumer safety, many countries and international bodies set MRL for antibiotic residues in foods of animal origin (Pavlína et al., 2011). The MRL represent the internationally accepted limits which specify the maximum or legally permitted quantity of drug residues that may be found in foodstuffs of animal origin (Kebede et al., 2014; Navrátilová, 2008). The MRL set by FAO/WHO (Codex Alimentarius Commission; CAC), the US Food and Drug Administration (USFDA), and the European Union (EU) are shown in Table 3.

Table 3: Internationally accepted maximum residue limits (MRL) for Antibiotics in milk according to FAO/WHO, the USFDA, and the EU.

Antibiotics	FAO/WHO (µg/kg)	EU (µg/kg)	USFDA (ppm)
Sulfamethoxine	0.01	_	0.01
Sulfathiazole	_	_	0.01
Sulfapyridine	_	_	0.01
Sulfaquinoxaline	_	_	0.01
Sulfanilamide	_	_	0.01
Trimethoprim	_	50	0.1
Gentamicin	200	100	0.1
Lincomycin	150	150	_
Ampicillin	_	4	0.01
Enrofloxacin	_	100	_
Penicillin G (Benzylpenicin)	4	4	0.01
Tetracycline	_	100	_
Oxytetracycline	100	100	0.1
Chlortetracycline	100	100	0.1
Tylosin	100	50	_

(Source: USFDA (2017); FAO/WHO (2015); EU (2009))

CHAPTER 3: MATERIALS AND METHODS

3.1 Study area

Kampala, the Capital City of Uganda is located in the south of the country, at an average elevation of 1,190 m (3.904 ft) above sea level. It lies at latitude 0° 20' 6 North and longitude 32° 34' 59 East. The resident population of Kampala is estimated to be 1936080 (0.78 male: 1.0 female) inhabitants of whom16% are children 5 to 9 years of age. Sixty percent of Kampala's population resides and or works within the peri-urban areas of the city (Bamuwamye et al., 2017). The mean annual precipitation is 46.22 inches (1174 mm) with peaks in May and November. The district is divided into five divisions namely Kampala Central, Nakawa, Rubaga, Kawempe and Makindye Divisions for management purposes. Because of its population demographics, Kampala Capital City has the highest number of milk selling points.

3.2 Sampling

A total of one hundred and twenty five samples (100 raw milk and 25 processed milk) were randomly obtained from different commercial milk centers in Kampala. Samples were separately placed in labeled sterile plastic bottles. They were then transported in ice boxes to the Directorate of Government Analytical Laboratories (DGAL) chemistry laboratory for analysis. Upon arrival at the laboratory, five samples from the same division were composited to represent a day's sample and stored at -18°C until analysis. This procedure was repeated for one day in a week over five weeks. Sampling was done between May and July 2017.

3.3 Materials and methods

3.3.1 Materials

3.3.1.1 Equipment

Antibiotic residues in milk were determined on an Agilent Liquid Chromatography-Tandem Mass Spectrometer (LC-MS/MS) (Agilent, Model 6420, United States). The 6420 triple quadrupole mass spectrometer operating in positive electrospray ionization (ESI) MS/MS mode was used for detection. Centrifugation of the samples was done using ROTOFIX (32A, Hettich GmbH, Germany). The Nitrogen evaporator (Athena technology, AT-EV-50, India) was used to concentrate the samples.

3.3.1.2 Chemicals and reagents

All chemicals used were analytical grade reagent. Methanol and acetonitrile were obtained from Fisher Scientific Limited in the United Kingdom. Standards (95% to 99.9% purity) of the antibiotics; quinolones (ciprofloxacin, enrofloxacin and nalidixic acid), tetracycline (tetracycline, oxytetracycline, chlortetracycline and metacycline), aminoglycosides (gentamicin), sulfonamides (sulfanilamide, sulfadiazine, sulfathiazole, sulfapyridine, sulfamerazine, sulfamethizole, sulfachloropyridazine, sulfamethoxazole, sulfamethoxine, sulfaquinoxaline, chloraminophenamide and sulfanitran), amphenicol (chloramphenicol) and ß-lactam (ampicillin and penicillin G were obtained from Sigma-Aldrich (The Netherlands).

3.3.2 Methods

3.3.2.1 Sample preparation

The sample milk (4 ml) was homogenised in a centrifuge tube for 10 minutes. Acetonitrile (6 ml) was added and the mixture vortexed for 30 seconds, followed by centrifugation at 3000 rpm for 10 minutes. The supernatant was transferred into a graduated glass tube. The glass tube was put into a water bath at 40°C under nitrogen flow and the extract evaporated to an end volume of 4 ml. With the use of syringe, the concentrated extract was filtered through a 0.22 µm filter and the filtrate transferred in a 2 ml auto sample vial. The filtrate (5 µl) was injected into the LC-MS/MS system.

3.3.2.2 Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Chromatographic separation was achieved using an Agilent C18 Zorbax eclipse column plus 2.1 × 100 mm × 1.8 mm at a temperature of 40°C. The mobile phase comprised 5 mM ammonium formate in a 50:50 water: methanol solvent regime, 0.1 formic acid in water (Mobile phase A), formic acid in acetonitrile and formic acid in methanol (Mobile phase B). The flow rate was kept constant at 0.5ml/min with an injection volume of 5 µl. The total run time was 27 minutes.

3.3.2.3 Gradient

Data was controlled and evaluated by Mass Lynx software (version 4.1). The selected reaction monitoring (MRM) mode was used and the following tune parameters were applied: capillary, 3 kV; cone 15 V; extractor, 3.00 V; source temperature, 150°C; desolvation temperature, 500°C, cone gas flow, $80Lh^{-1}$; desolvation gas flow, $1000Lh^{-1}$; collision gas flow, 0.16 mL min^{-1},

resolution (LM1, HM1, LM2, HM2 where LM is low mass and HM is high mass), 2.7, 15, 2.8, 14.8; ion energy (1 and 2), 0.3, 0.6; multiplier 546.52 V. Fragment voltage (V), collision energy (eV) and transition mass parameters for all antibiotic residues analyzed in milk samples are presented in Table 4.

Table 4: Mass spectrometry parameters used for the screening of antibiotic residues in milk

Antibiotics	Transition	Voltage (V)	Collision energy (V)
Sulfanilamide	173.0 > 92.0	150	40
Sulfadiazine	251.1 > 156.0	150	20
Sulfathiazole	260 > 156	150	20
Sulfapyridine	250 > 56.0	150	20
Sulfamerazine	265.1 > 92.0	150	40
Sulfamethizole	271.0 > 156.0	150	40
Sulfachloropyridazine	285.0 > 156.0	150	40
Sulfamethoxazole	241.1 > 92.0	150	20
Sulfaquinoxaline	301.1 > 92. 0	150	40
Sulfadimethoxine	311.1 > 156.1	150	40
Sulfanitran	334.1 > 136.0	150	40
Tetracycline	445.2 > 410.1	150	20
Doxycycline	445.2 > 428.1	200	20
Oxytetracycline	461.2 > 426.1	150	20
Chlortetracycline	479 > 462.1	150	20
Metacycline	443.1 > 426.1	150	40
Ciprofloxacin	332.1 > 314.1	150	40
Enrofloxacin	360.2 > 342.2	150	20
Penicillin G	335.1 > 160.0	150	10
Ampicillin	348.1 > 207.1	150	40
Nalidixic	233.1 > 215.1	150	20
Gentamicin	478.3 > 157.1	150	20
Chloraphenical	321.0 > 257.0	150	10
Lincomycin	407.2 > 126.1	150	40
Tylosin	916.5 > 174.1	150	40
Chloraminophenamide	284.0 > 78.0	150	40

3.4 Data Analysis

Statistical analysis was carried out using one-way ANOVA to determine if there were any significant differences in levels of the subgroups of antibiotic residues within divisions and among the different types of milk with graph pad 57. The significant level was considered at $p<0.05$.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Levels of sulphonamide residues in milk

Chloraminophenamide (4-amino-6-chloro-1, 3-benzenedisulfonamide) and sulfathiazole were present in relatively high amount in both processed and raw milk (Table 5). The amounts in raw and processed milk ranged from 3.3856 to 15.7146 µg/l and 0.0164 to 0.0437 µg/l, respectively. However, these levels were not significantly different in raw and processed milk. Sulfachloropyridazine, sulfanitran, sulfamethoxazole and sulfadiazine were detected in trace levels in both processed and raw milk. Sulfanilamide, sulfapyridine, sulfamerazine and sulfadimethoxine were only present in processed milk. Sulfamethizole and sulfaquinoxaline were not detected in both processed and raw milk.

Levels of Chloraminophenamide and Sulfathiazole in milk from Makindye, Rubaga, Kawempe, Kampala Central and Nakawa did not present any statistical difference. Sulfathiazole was detected in that amount in Kawempe but was relatively low in raw milk from Nakawa (Table 5). Processed milk had similar levels as raw milk. Pasteurization and other forms of heat treatment have limited effects on antibiotic residues in milk (Zorraquino et al., 2009). This study showed that sulfathiazole levels were in line with those obtained by Darko et al. (2017) for raw milk in developing countries. However, the levels of sulfonamide antibiotic residues in milk were lower than reported elsewhere in Africa (Table 6). They were also lower than the maximum residue limit set for these antibiotics in milk by FAO/WHO (2015), USFDA (2017) and the EU (2009).

Detected amounts of antibiotic residues in milk could be an indicator of farmers' non-observance of withdrawal periods in lactating animals (Orwa et al., 2017). It could also mean that animals

are fed on antibiotic contaminated feeds or milk deliberately adulterated with antibiotics for purposes of extending its shelf life. Antibiotic residues in milk may result in allergenic reactions in humans, and may in the long run facilitate the development of resistant pathogens (Forouzan et al., 2014). Sulfonamide drugs are commonly used as growth promoters and as therapeutic and prophylactic drugs in food producing animals (Darko et al., 2017).

Table 5: Levels (µg/l) of sulfonamide residues in processed and raw milk from five divisions of Kampala City

		Raw Milk				
Antibiotics	Processed milk	Kampala Central	Nakawa	Rubaga	Makindye	Kawempe
Chloraminophenamide	9.5884 ± 9.8441^{b}	7.4512 ± 3.6119^{b}	3.3856 ± 2.4929^{b}	9.5861 ± 7.2781^{b}	15.7146 ± 16.7871^{b}	8.5584 ± 10.2556^{b}
Sulfathiazole	0.0191 ± 0.0103^{a}	0.0379 ± 0.0251^{a}	0.0164 ± 0.0039^{a}	0.0183 ± 0.0203^{a}	0.0244 ± 0.0265^{a}	0.0437 ± 0.0451^{a}
Sulfachloropyridazine	0.001 ± 0.0009^{a}	0.0002 ± 0.0001^{a}	0.0004 ± 0.0002^{a}	0.0002 ± 0.0001^{a}	0.023 ± 0.0327^{a}	0.0014 ± 0.0013^{a}
Sulfanitran	0.0011 ± 0.0010^{a}	0.0007 ± 0.0003^{a}	0.0011 ± 0.0007^{a}	0.0026 ± 0.0032^{a}	0.0009 ± 0.0004^{a}	0.0051 ± 0.0013a
Sulfamethoxazole	0.0014 ± 0.0018^{a}	0.0002 ± 0.0001^{a}	0.0002 ± 0.0001^{a}	0.0002 ± 0.0001^{a}	0.0002 ± 0.0001^{a}	0.0046 ± 0.0033^{a}
Sulfadiazine	0.0006 ± 0.003^{a}	0.0001 ± 0.0001^{a}	0.0002 ± 0.0001^{a}	ND	0.0002 ± 0.0001^{a}	ND
Sulfanilamide	0.0020 ± 0.0022^{a}	ND	ND	0.0002 ± 0.0001^{a}	0.0001 ± 0.0001^{a}	ND
Sulfapyridine	0.0014 ± 0.0012^{a}	ND	ND	ND	ND	ND
Sulfamerazine	0.0006 ± 0.0002^{a}	ND	ND	ND	ND	ND
Sulfadimethoxine	0.0002 ± 0.0002^{a}	ND	ND	ND	ND	ND
Sulfamethizole	ND	ND	ND	ND	ND	ND
Sulfaquinoxaline	ND	ND	ND	ND	ND	ND

Mean concentration with different superscript letters are significantly different across rows at $p = 0.05$

Table 6: Levels (µg/l) of antibiotic residues in milk from developing countries

Antibiotics	Uganda - Raw[1]	Uganda - Processed[1]	Ghana[2]	Kenya[3]	Algeria[4]	Ethiopia[5]	Nigeria[6]
Sulfathiazole	0.0028 - 0.0949	0.0064 - 0.0332	0.014	ND	1,400	-	-
Sulfamethoxazole	0.0001 - 0.0069	0.0001 - 0.0038	0.25	8,979	2,100	-	-
Sulfadiazine	ND	0.0001 - 0.0004	-	90.03	-	-	-
Sulfadimethoxine	0.0045 - 0.015	0.0001 - 0.0004	-	66.14	-	-	-
Sulfachloropyridazine	0.0001 - 0.057	0.0002 - 0.0021	-	-	3,000	-	-
Sulfamerazine	ND - 0.0008	0.0005 - 0.0009	-	-	1,300	-	-
Sulfapyridine	ND - 0.0056	0.0001 - 0.003	-	-	1,200	-	-
Sulfaquinoxaline	0.0018 - 0.0163	ND–0.0013	-	-	800	-	-
Penicillin G	ND- 0.0001	ND - 0.0001	-	-	-	0 - 47	6.18 - 16.76
Chloramphenicol	0.0001 - 0.0011	0.0001 - 0.0006	0.12	-	-	-	-
Oxyteracycline	0.0001 - 0.0008	0 - 0.0001	0.25	-	-	27 - 251	-

(Source: 1.This study; 2. Darco, et al. (2017); 3. Orwa1 & Lamuka (2017); 4. Layada & Andjelkovic (2016); 5. Abebew (2014); 6. Olatoye & Ishola (2016)).

4.2 Levels of tetracycline residues in milk

Chlortetracycline and metacycline were detected in trace levels in both processed and raw milk from Kampala while doxycycline, oxytetracycline and tetracycline were hardly detected (Figure 12). The concentration of chlortetracycline was notably high. Makindye presented the highest concentration of chlortetracycline in milk while Kampala Central had the least concentration. Chlortetracycline was observed to be present in higher levels in processed milk than in raw milk. Oxytetracycline levels were lower than reported for raw milk in Ghana (Darko et al. 2017) and Ethiopia (Abebew et al., 2014). In general, tetracycline residues were lower than the MRL set for these antibiotics by FAO/WHO (2015), USFDA (2017) and the EU (2009). Levels of tetracycline antibiotics in milk are indicative of their use in animal treatment in Uganda. Tetracyclines are teratogenic, cause allergenic reactions in sensitive persons and increase the risk of antimicrobial resistance in humans and animals (Elbagory et al. 2017; Orwa1 et al. 2017).

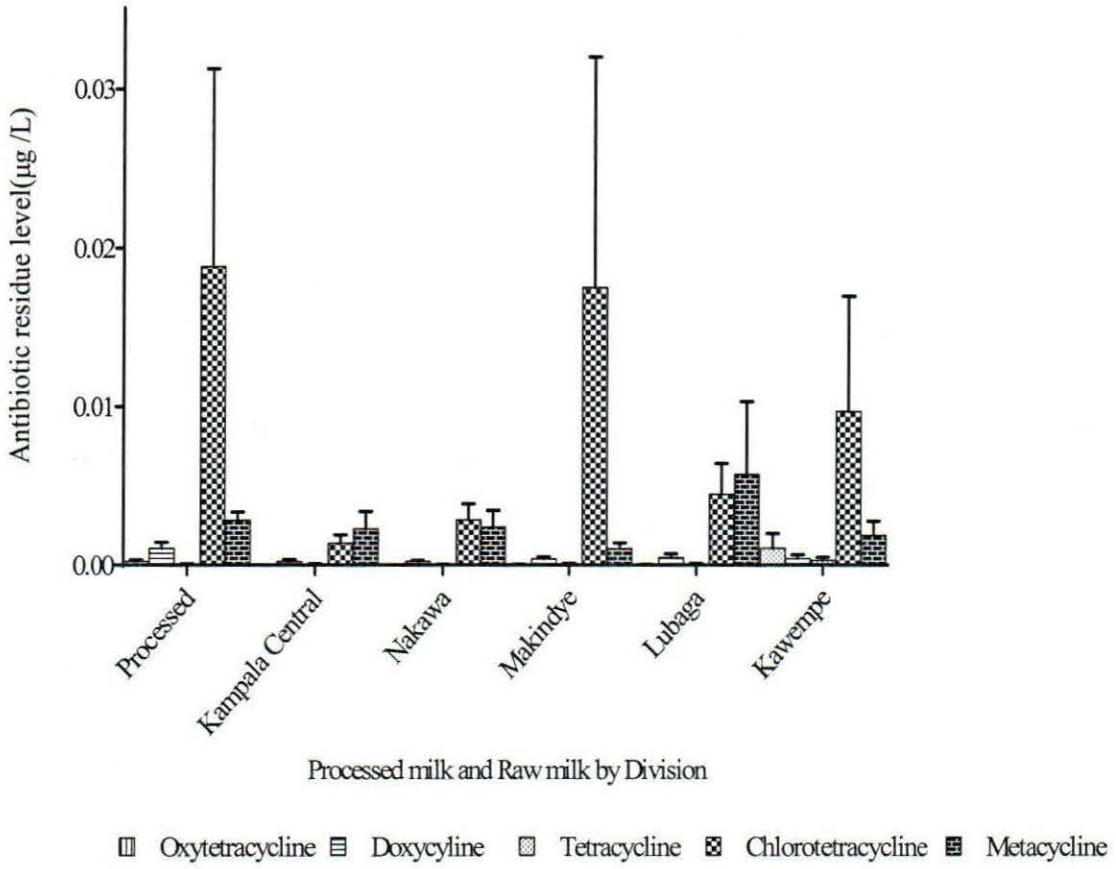

Figure 12: Levels (µg/l) of tetracycline residues in milk

4.3 Levels of quinolones (fluoroquinolones) residues in milk

In both processed and raw milk, the concentration of ciprofloxacin was notably high (Figure 13). Enrofloxacin was detected in trace levels in processed and raw milk from Makindye and Kawempe. Milk from Kawempe tested positive for nalidix acid antibiotics. Kawempe Division also had the highest value (0.0221 µg/l) of ciprofloxacin in raw milk while Rubaga Division had the lowest value (0.0066 µg/l). Enrofloxacin and nalidix acid hâd ranges of not detected to 0.0076 µg/l and not detected to 0.0075 µg/l, respectively. There are no established MRL for nalidix acid and ciprofloxacin. In addition, studies on ciprofloxacin residue levels in milk in

Africa are limited. However, ciprofloxacin is often recommended for respiratory tract, gastrointestinal tract infections and urinary tract infections that are caused by *Campylobacter, E. coli, Haemophilus, Mycoplasma, Pasteurella* and *Salmonella species* in animals (Khan et al. 2015). Quinolones show damage to the juvenile joint, kidney, eye and the central nervous system (Buket et al.2013.)

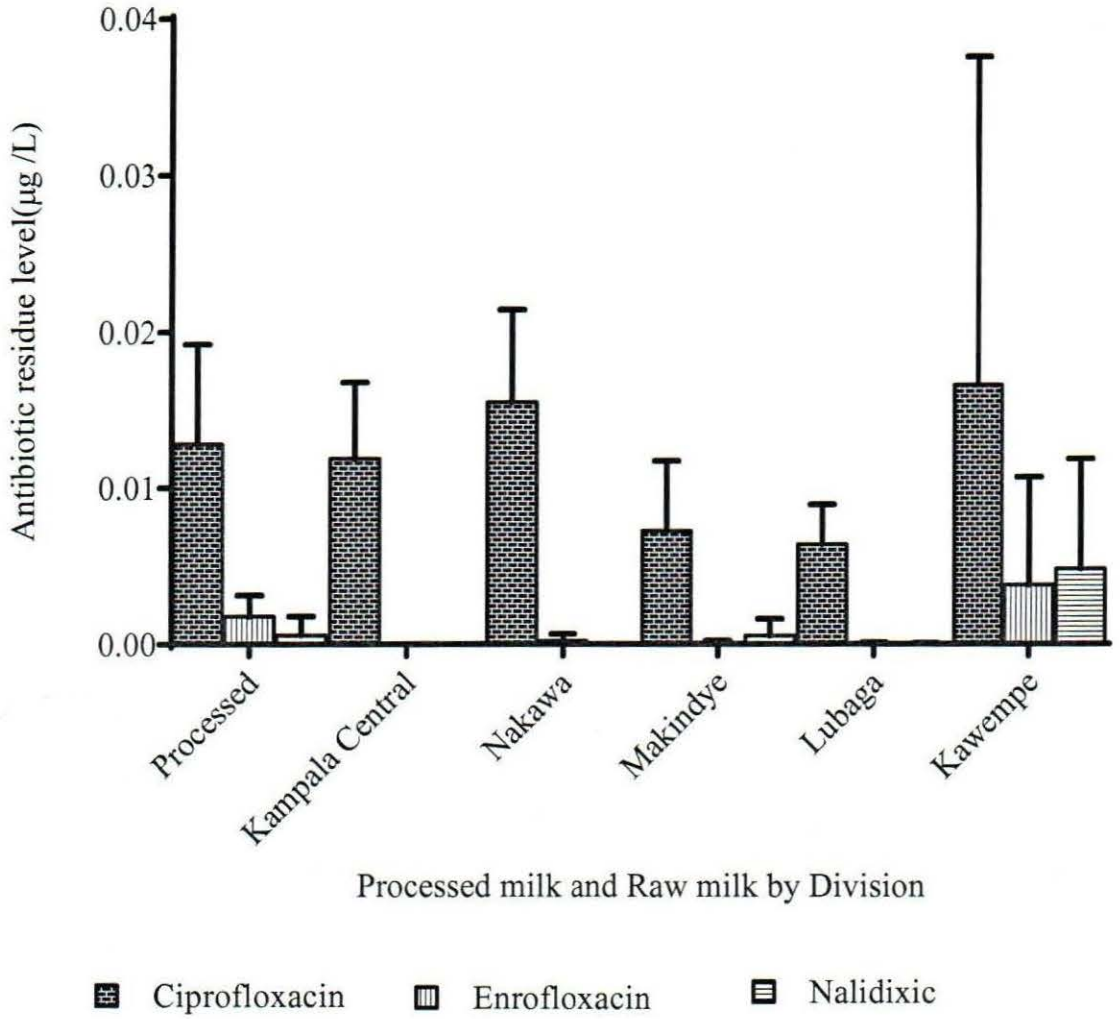

Figure 13: Levels (µg/l) of quinolone residues in milk

4.4 Levels of beta-lactam residues in milk

Of the two β –lactam drugs analysed in this study, only ampicillin was detected in both processed and raw milk (Figure 14). Ampicillin levels ranged from 0.044 to 0.216 µg/l in raw milk while processed milk had a concentration of 0.0594 µg/l. There was no observable difference in ampicillin levels among the two milk types.

Raw milk from Makindye registered the highest concentration while the lowest concentration was recorded in Kampala Central. These results indicate that processing of milk had limited effect on antibiotic residue levels. Ampicillin and Penicillin G (Benzyl penicillin) residues were observed to be lower in Ugandan milk than reported in Ethiopia and Nigeria (Table 6). Their levels were also lower than the USFDA and EU limits of 0.01 ppm and 4 µg/kg, respectively, for these antibiotics in milk for human consumption. Beta lactam antibiotics are widely used in the treatment of mastitis in cows (Rama et al., 2017). Non-observance of withdrawal period has been linked to the presence of β-lactam antibiotic residues in food (Boamah et al., 2016).

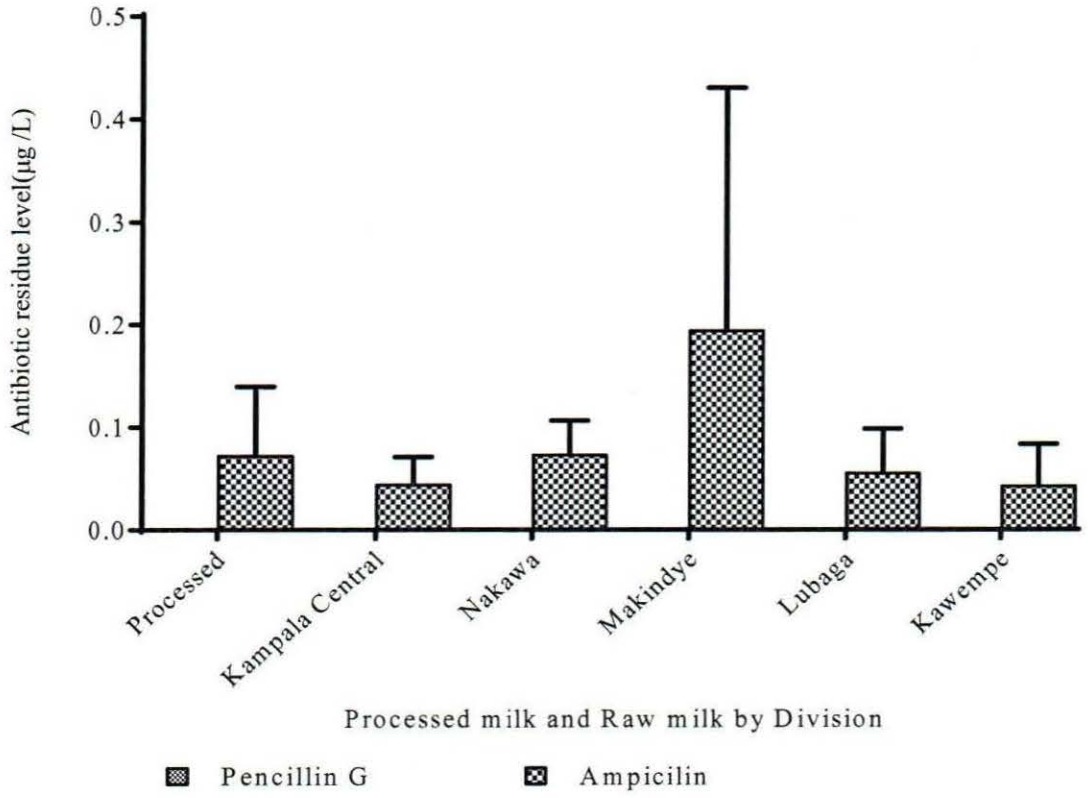

Figure 14: Levels (µg/l) β-lactam residues in milk

4.5 Levels of aminoglycosides residues in milk

The levels of the aminoglycoside, gentamycin in both raw and processed milk were found to be higher than those of all the other antibiotics considered in this study (Figure 15). Nakawa Division had the highest value (15.9844 µg/l) and Kawempe had the lowest value (4.6985 µg/l). There was no observed difference in the levels of gentamycin in processed and raw milk. There was as well, no difference in gentamycin levels with in divisions. The residue levels of gentamycin observed in this study were below the MRL of 200 µg/kg, 100 µg/kg and 0.1 ppm set by FAO/WHO (2015), EU (2009) and USFDA (2017), respectively. Gentamycin is nephrotoxic and causes immune deficiencies in humans (Tan et al. 2009). Indiscriminate use of gentamycin

while not respecting withdrawal periods can lead to a buildup of its residues to high levels in cow's milk (Layada et al. 2016).

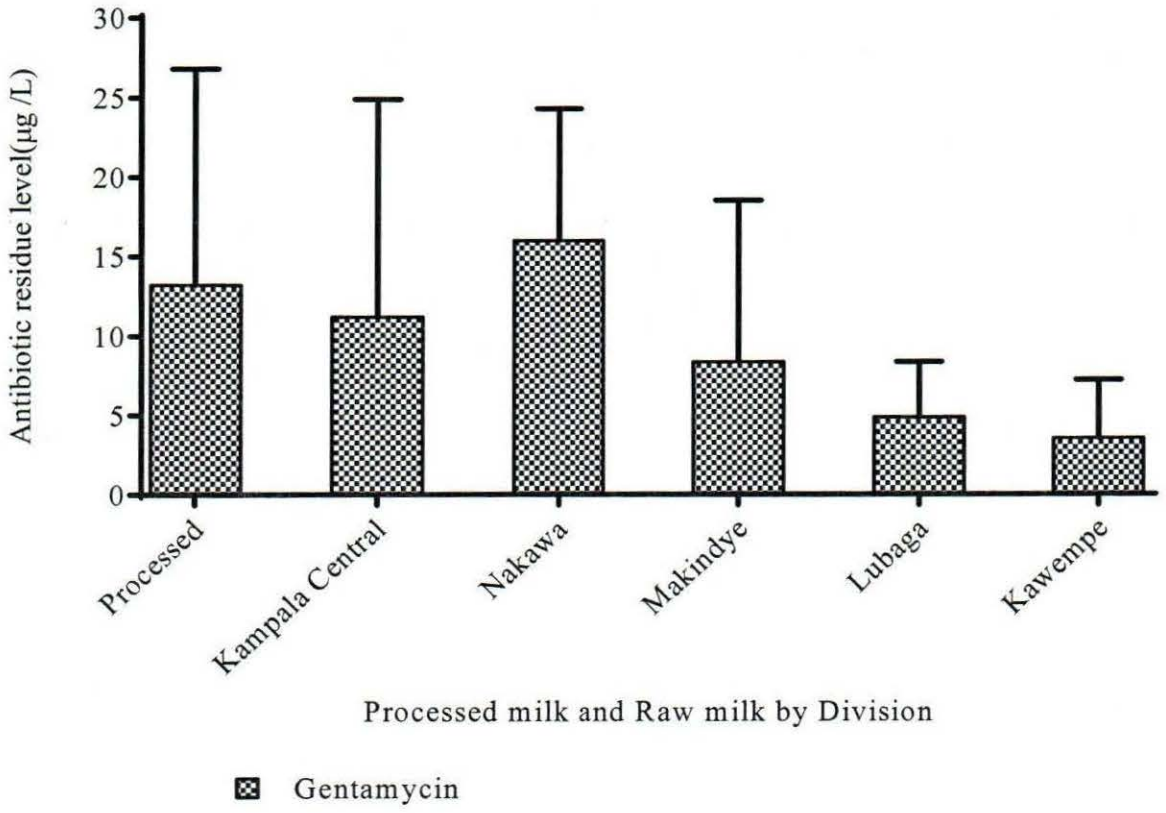

Figure 15: Levels (µg/l) aminoglycosides residues in milk

4.6 Levels of amphenicol, lincomycin and macrolide residues in milk

Chloramphenicol, tylosin and lincomycin were detected in very low levels. Chloramphenicol was taken to represent the phenicol group and its levels ranged from not detected to 0.0003 µg/l (Figure 16). There was no pronounced difference ($P>0.05$) between chloramphenicol levels in processed and raw milk. Processed milk had levels of 0.0003 µg/l while for raw milk the levels were 0.0002, 0.0001 and 0.0001 ± 0.0001 µg/l for Kawempe, Makindye and Kampala Central,

respectively. There is no MRL set for chloramphenicol in milk but the results of this study showed lower chloramphenicol levels than previously reported for raw milk in Ghana (Darko et al., 2017).

Tylosin and erythromycin are the main macrolide antibiotics used in animal production. Tylosin was present in only two raw milk samples obtained from Makindye and Kawempe divisions, with concentrations of 0.0004 µg/l and 0.0038 µg/l, respectively. Tylosin residues were also below international maximum residue limits. The lincosamide, lincomycin was present in only processed milk with concentration of 0.0026 µg/l, which was below the MRL according to FAO/WHO (2015), USFDA (2017) and the EU (2009).

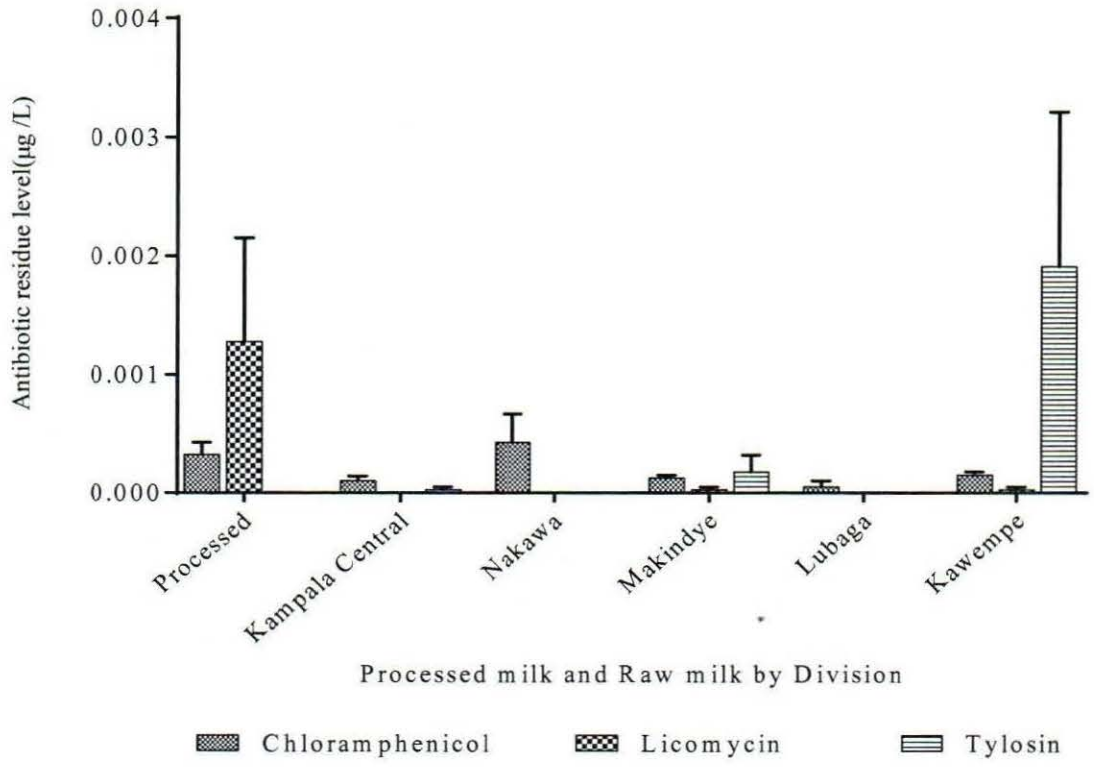

Figure 16: Levels (µg/l) amphenicol, licomycin and macrolide residues in milk

CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Processed and raw milk from Kampala contained safe levels of antibiotics residues, the levels were below international maximum residue limits. Variation in residue levels in processed and raw milk did not present considerable differences. No variation was notable in milk from different divisions. Gentamicin and chloraminophenamide were present in high levels in milk. Sulfathiazole, ampicillin, ciprofloxacin and chlortetracycline were also detected.

5.2 Recommendations

However, given the health risk associated with chronic exposure to antibiotics in food, there is need for regular monitoring of milk in Kampala for antibiotic residues as a means of consumer protection. Overall, milk in Uganda is safe for human consumption given that the concentration of the antibiotic residues were below the international maximum residue limits.

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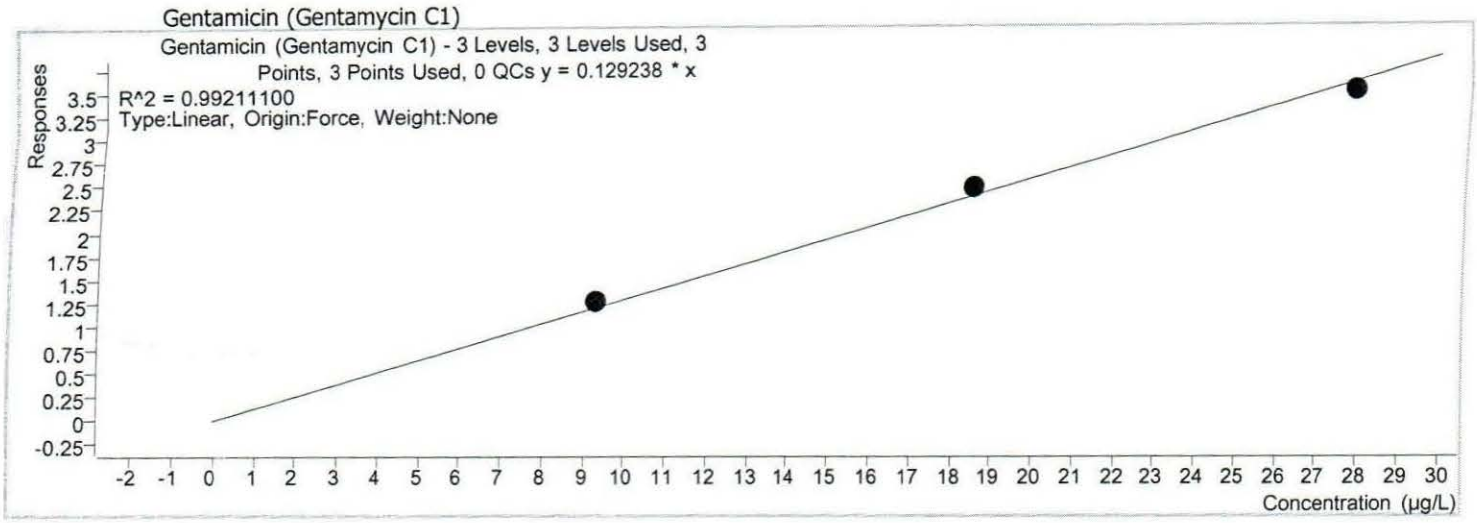
APPENDICES

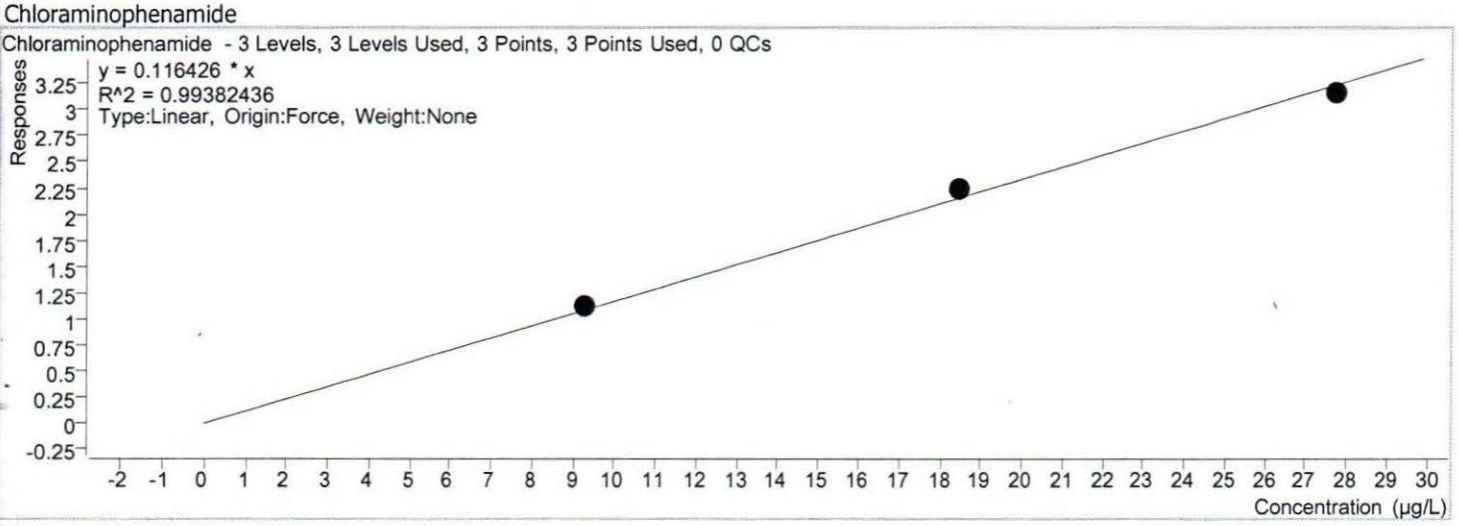
Appendix 1: Calibration curve for Gentamicin standard solution



Calibration STD Path	Cal Type	Level	Enabled	Resp.	Exp. Conc	Resp. Factor
D:\MassHunter\Data\Antibiotics\07-07-2017 Antibiotics Std 1.d	Calibration	1	x	1	9.3000	0.1378
D:\MassHunter\Data\Antibiotics\07-07-2017 Antibiotics Std 2.d	Calibration	2	x	2	18.5000	0.1336
D:\MassHunter\Data\Antibiotics\07-07-2017 Antibiotics Std 3.d	Calibration	3	x	4	27.7500	0.1263

Appendix 2: Calibration curve for chloraminophenamide standard solution

Chloraminophenamide



Calibration STD Path	Cal Type	Level	Enabled	Resp.	Exp. Conc	Resp. Factor
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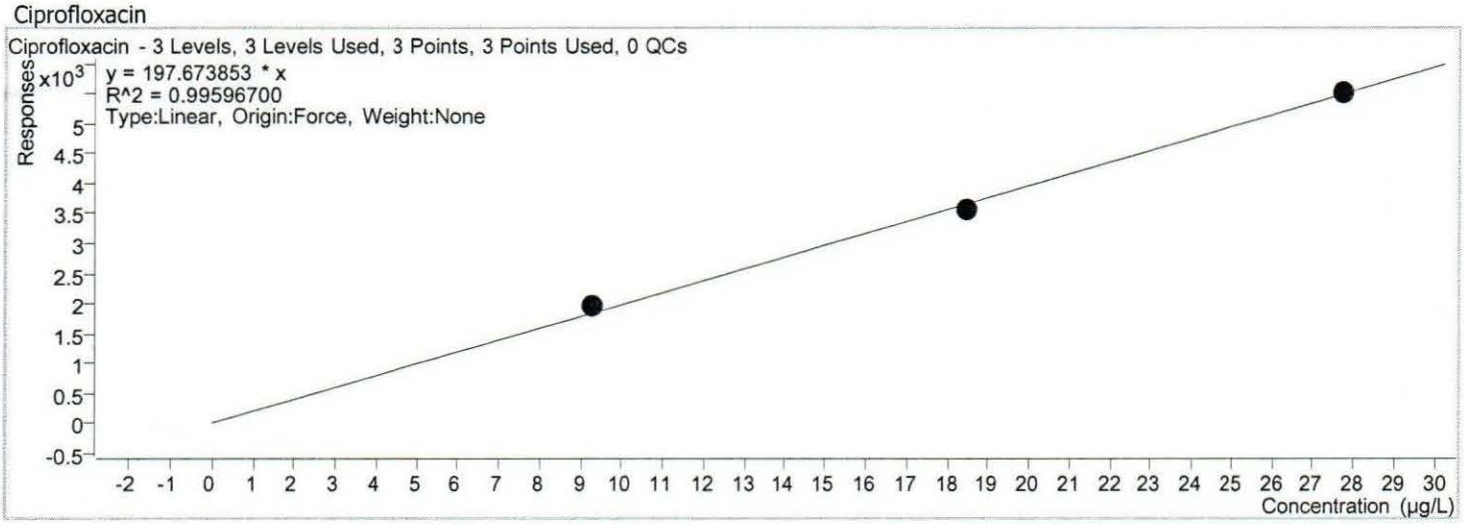
D:\MassHunter\Data\Antibiotics\07-07-2017 Antibiotics Std 2.d	Calibration	2	x	2	18.5000	0.1207
D:\MassHunter\Data\Antibiotics\07-07-2017 Antibiotics Std 3.d	Calibration	3	x	3	27.7500	0.1140

Appendix 3: Calibration curve for ciprofloxacin standard solution

Ciprofloxacin



Calibration STD Path	Cal Type	Level	Enabled	Resp.	Exp. Conc	Resp. Factor
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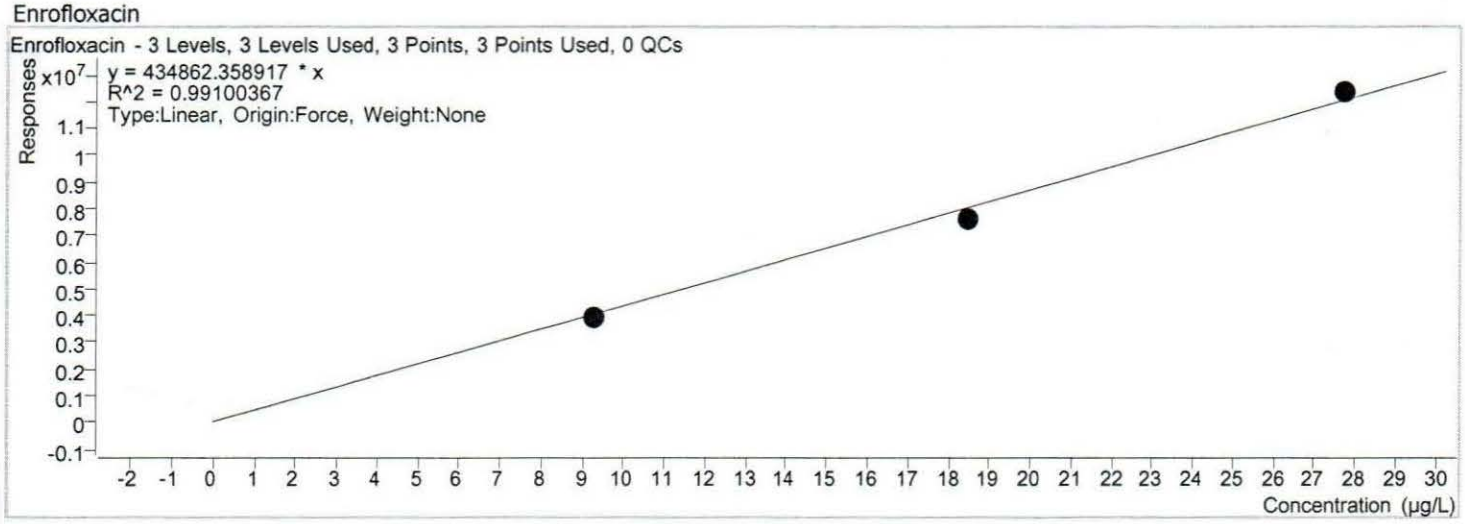
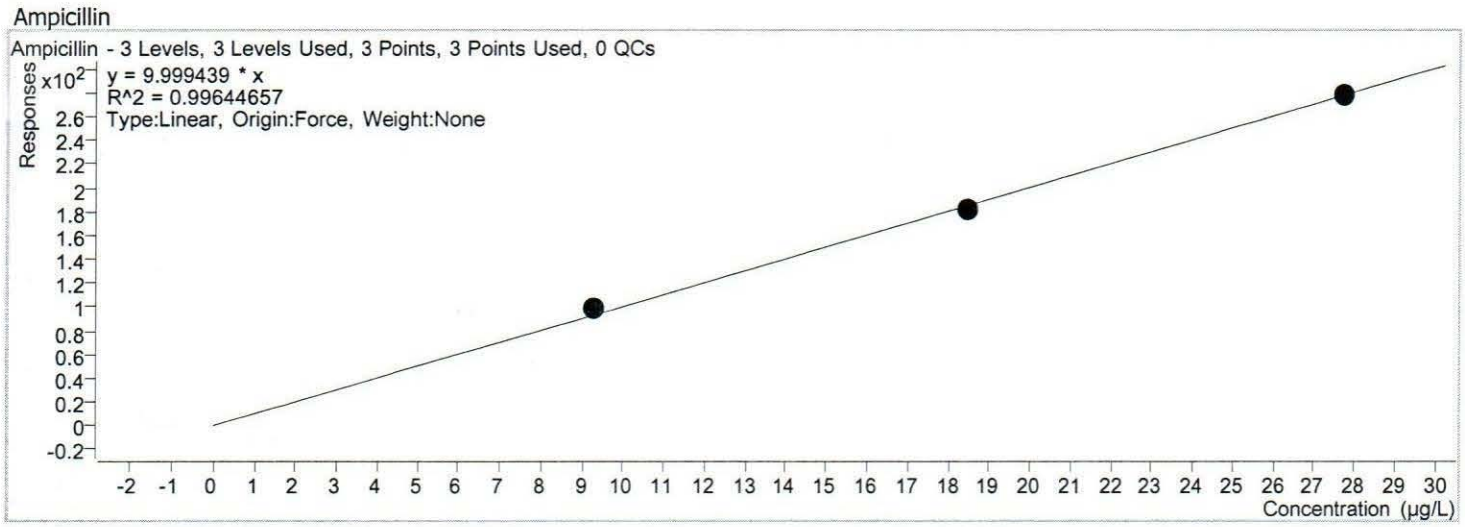
D:\MassHunter\Data\Antibiotics\07-07-2017 Antibiotics Std 2.d	Calibration	2	x	3548	18.5000	191.7881
D:\MassHunter\Data\Antibiotics\07-07-2017 Antibiotics Std 3.d	Calibration	3	x	5520	27.7500	198.9279

Appendix 4: Calibration curve for Enrofloxacin standard solution



Calibration STD Path	Cal Type	Level	Enabled	Resp.	Exp. Conc	Resp. Factor
D:\MassHunter\Data\Antibiotics\07-07-2017 Antibiotics Std 1.d	Calibration	1	x	3912770	9.3000	420727.9538
D:\MassHunter\Data\Antibiotics\07-07-2017 Antibiotics Std 2.d	Calibration	2	x	7603762	18.5000	411014.1772
D:\MassHunter\Data\Antibiotics\07-07-2017 Antibiotics Std 3.d	Calibration	3	x	12405612	27.7500	447049.0644

Appendix 5: Calibration curve for Ampicillin standard solution



Calibration STD Path	Cal Type	Level	Enabled	Resp.	Exp. Conc	Resp. Factor
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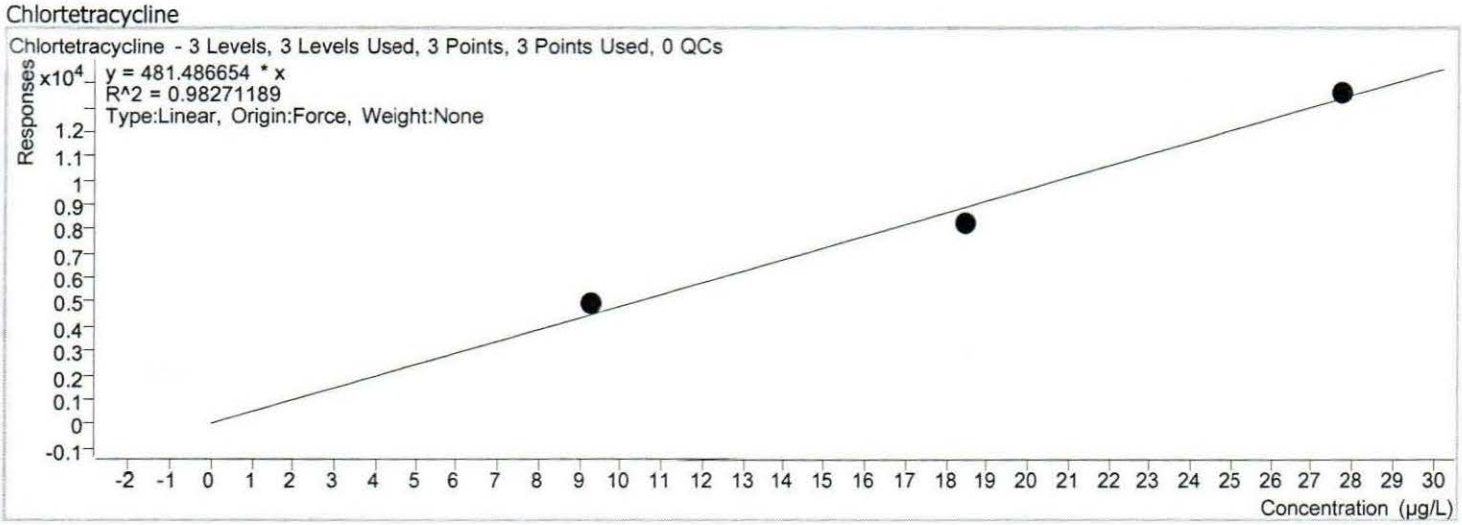
D:\MassHunter\Data\Antibiotics\07-07-2017 Antibiotics Std 2.d	Calibration	2	x	181	18.5000	9.7701
D:\MassHunter\Data\Antibiotics\07-07-2017 Antibiotics Std 3.d	Calibration	3	x	278	27.7500	10.0264

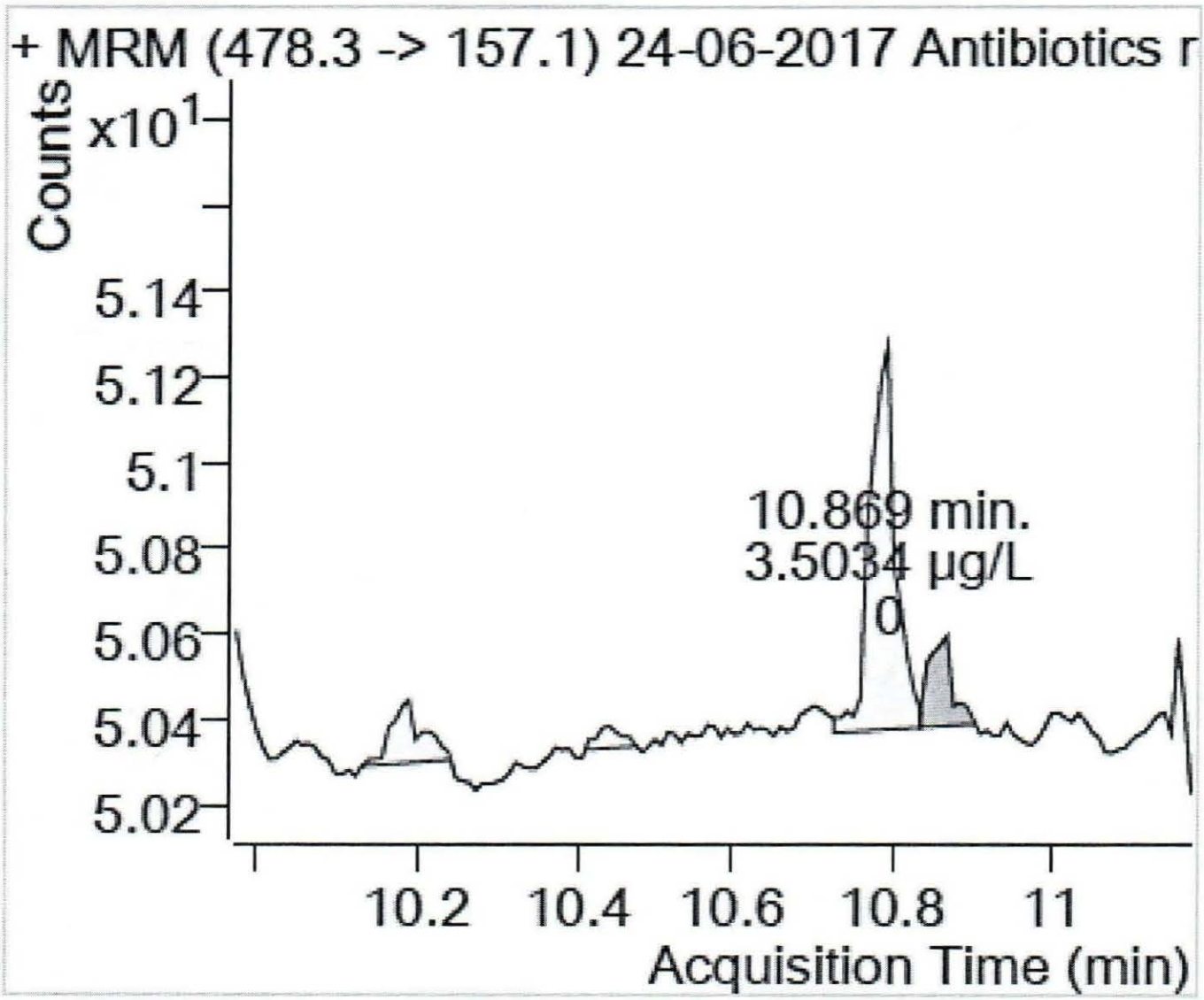
Appendix 6: Calibration curve for chlortetracycline standard solution

Chlortetracycline

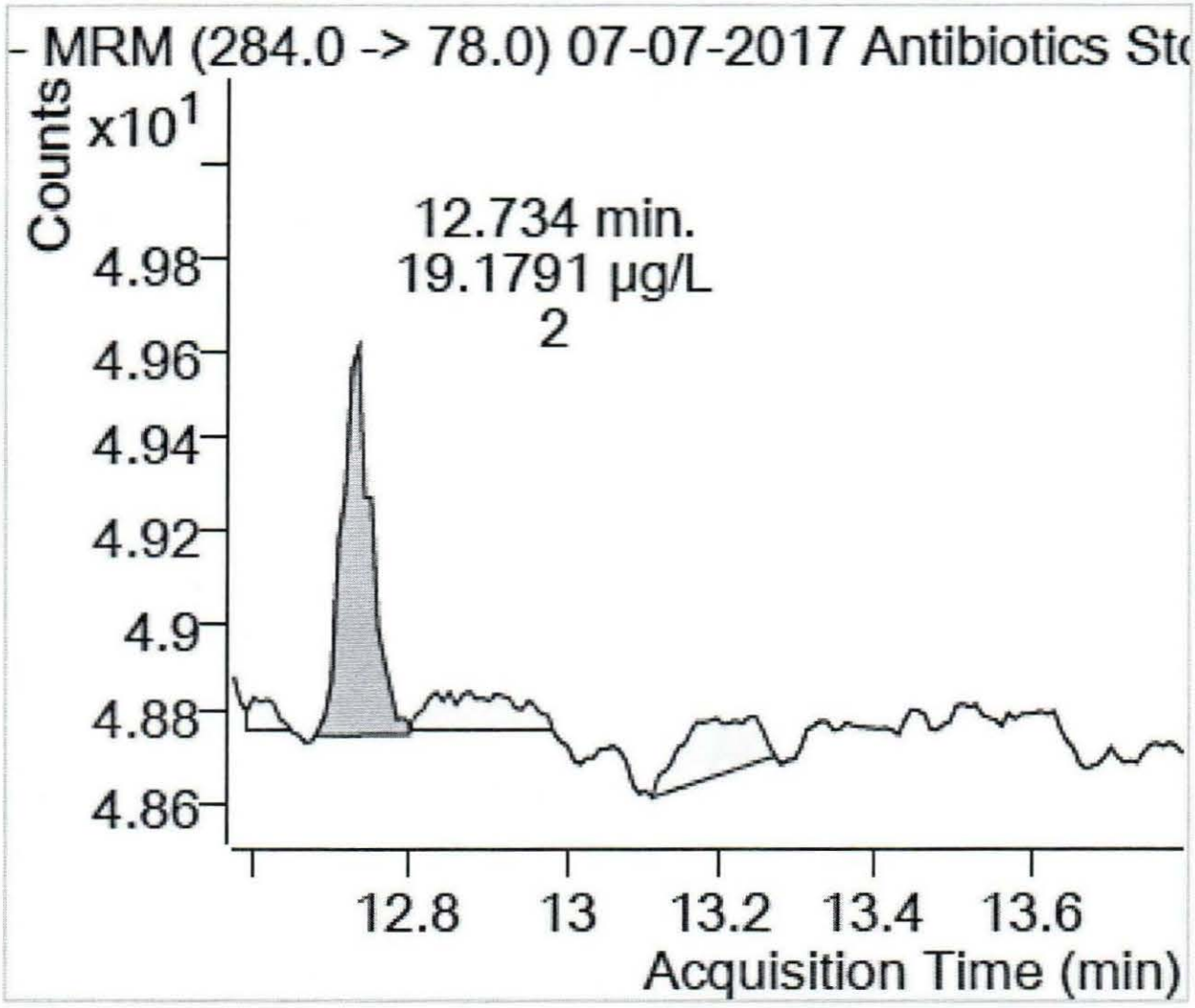


Calibration STD Path	Cal Type	Level	Enabled	Resp.	Exp. Conc	Resp. Factor
D:\MassHunter\Data\Antibiotics\07-07-2017 Antibiotics Std 1.d	Calibration	1	x	4944	9.3000	531.5973
D:\MassHunter\Data\Antibiotics\07-07-2017 Antibiotics Std 2.d	Calibration	2	x	8292	18.5000	448.1930
D:\MassHunter\Data\Antibiotics\07-07-2017 Antibiotics Std 3.d	Calibration	3	x	13616	27.7500	490.6556

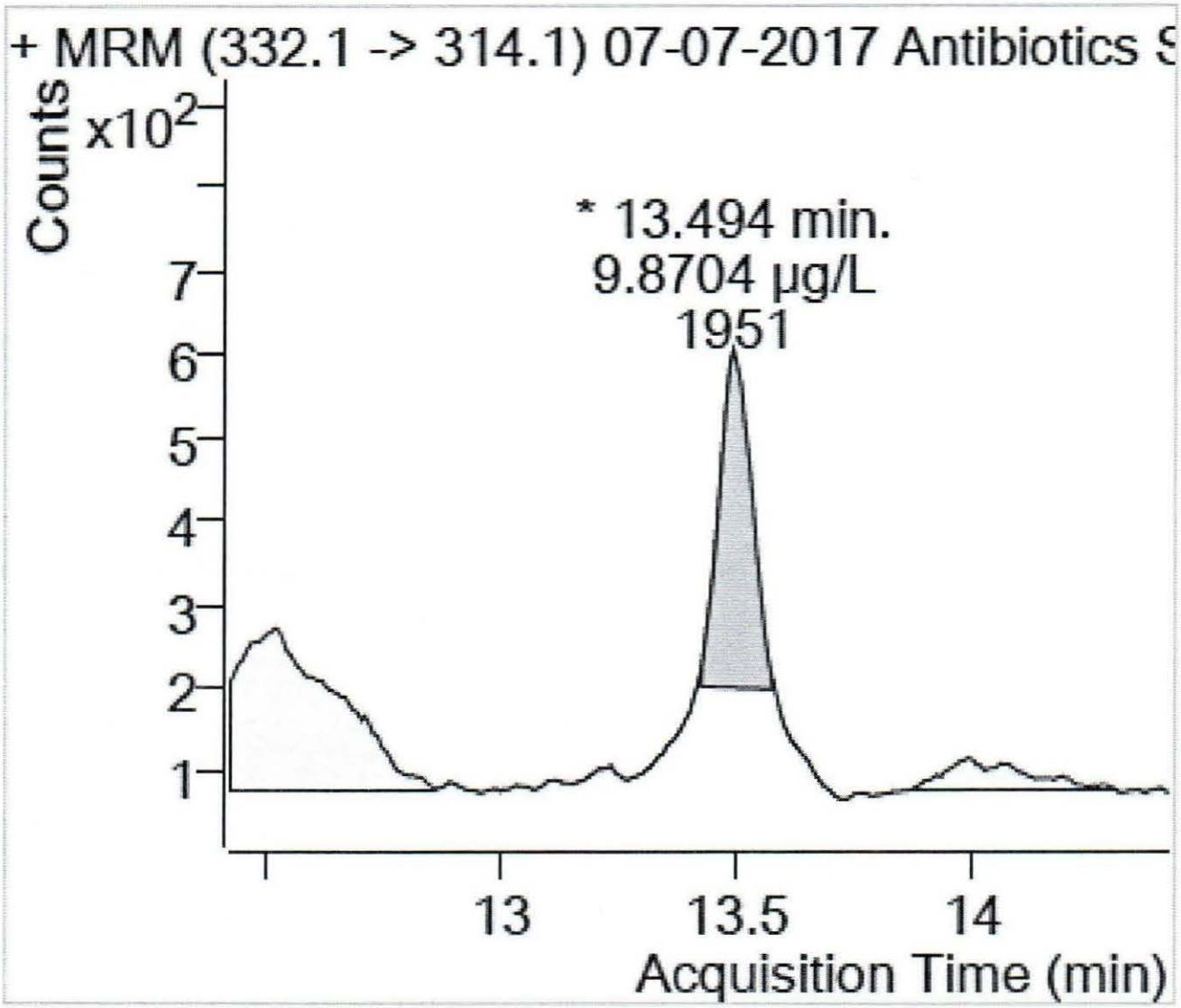
Appendix 7: Chromatogram of milk sample with Gentamicin residues



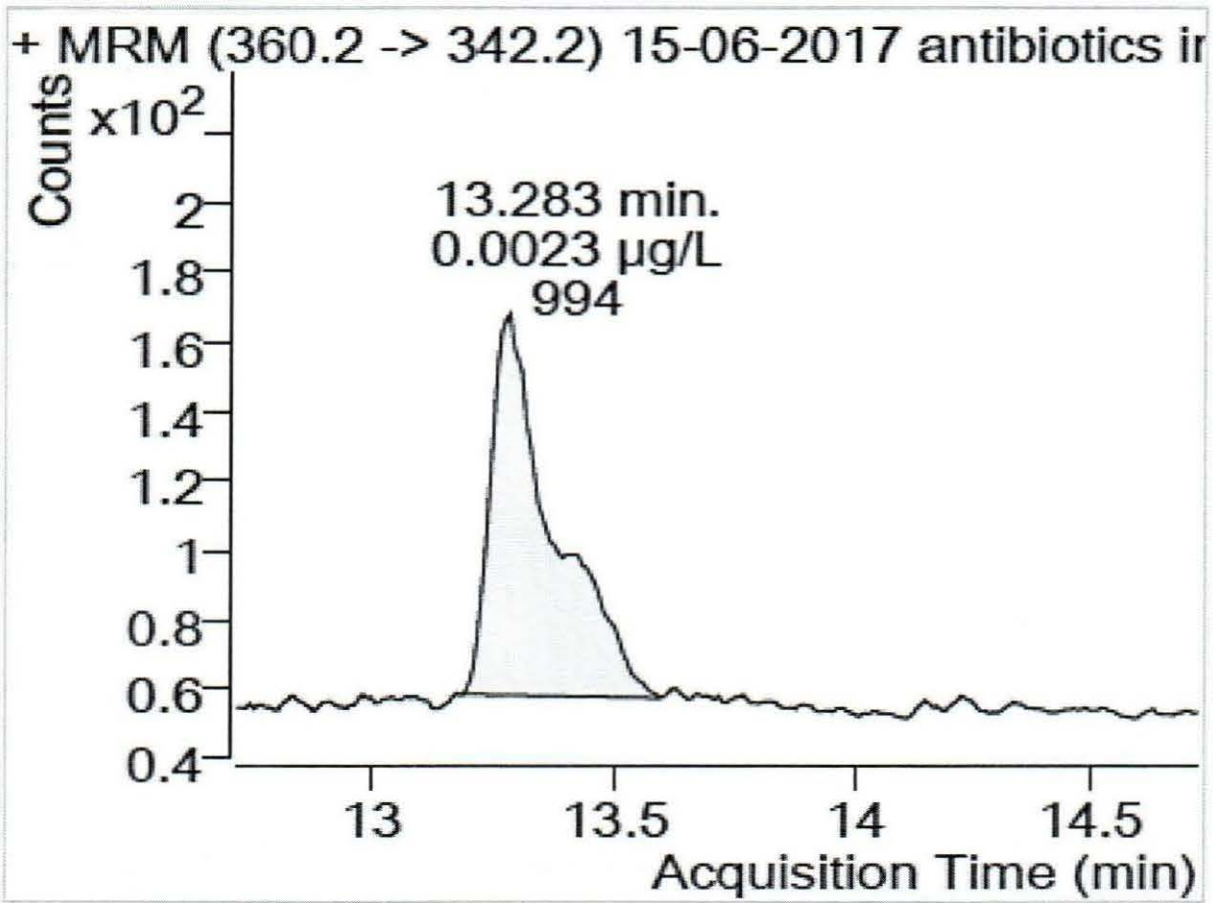
Appendix 8: Chromatogram of milk sample with chloraminophenamide residues



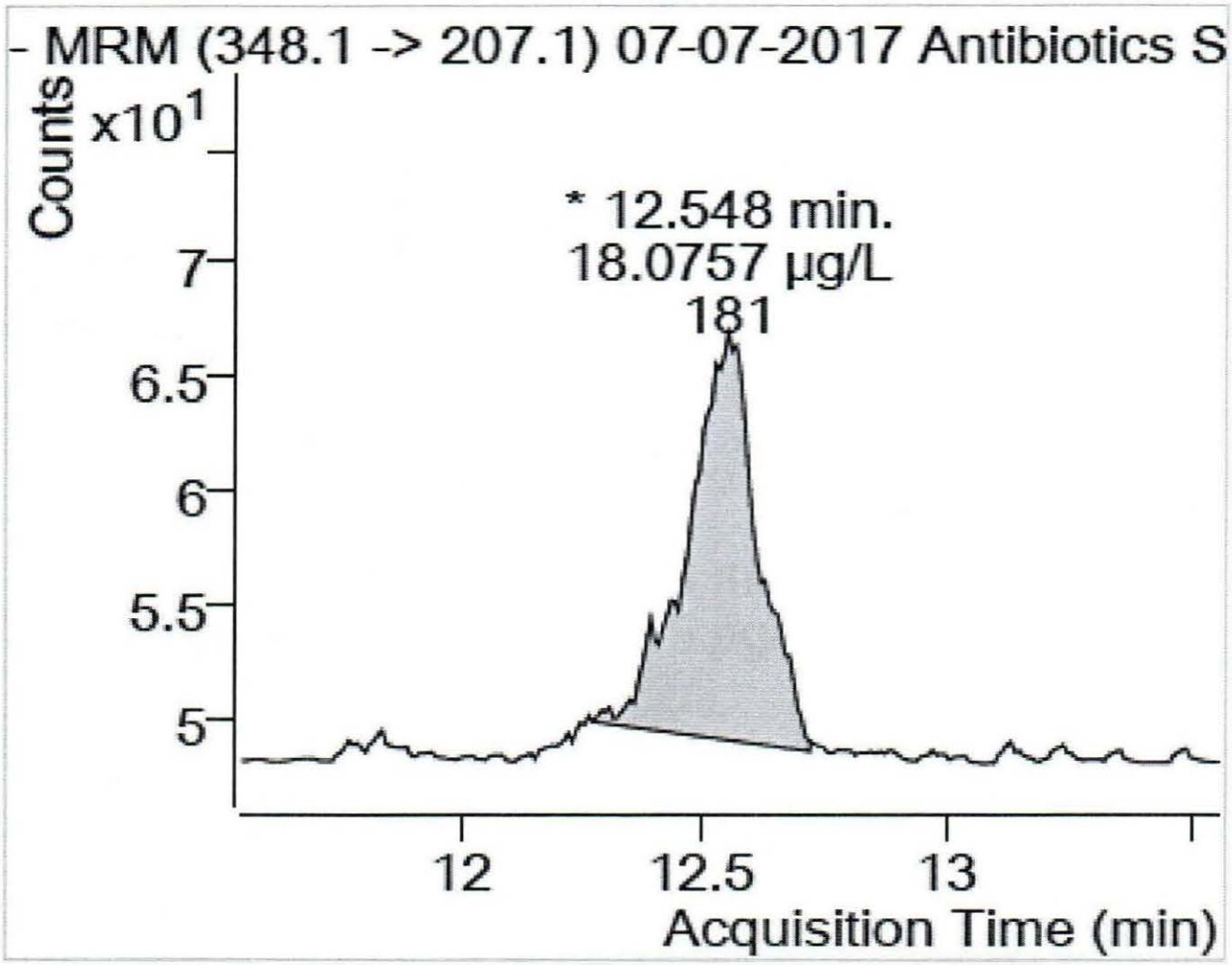
Appendix 9: Chromatogram of milk sample with ciprofloxacin residues



Appendix 10: Chromatogram of milk sample with Enrofloxacin residues



Appendix 11: Chromatogram of milk sample with Ampicillin residues



Appendix 12: Chromatogram of milk sample with chlortetracycline residues