

**Occurrence of antibiotic residues in milk. A case  
study of Gweru 2014.**

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**Occurrence of antibiotic residues in milk. A case study of Gweru.**

BY

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## ABSTRACT

The aim of the research was to determine the occurrence of antibiotic residues in milk in Gweru. The population consisted of twenty-eight farmers. Ten farmers were randomly selected from the population. The average volume that the farmers supply to dairy processing companies was twenty thousand liters during the time of sampling. Raw milk was sampled in two hundred and fifty mls sterile sample bottles. The samples were frozen up to the time of analysis. Detection and quantitation of antibiotic residues was done using High Performance Liquid Chromatography coupled to a mass spectroscopy detector (HPLC-MS). Fourteen antibiotics were analyzed for and these are: the sulphonamides, (trimethoprin, sulfaquinoxali, sulfamethoxin, sulfamethoxazole, sulfamethizole, sulfamethazine and sulfachloropyril), the steroids dexamethasone, the flouroquinolones which is enrofloxacin, the  $\beta$ -Lactams which is Penicillin G and amoxicillin as well as the Benzimidazoles which are thiabendazole and albendazole. Only one antibiotic which is albendazole was detectable on the two analyses which were done separately on the qualitative HPLC-MS machine in all the samples. The quantities of the albendazole were then quantified on the Quantitative HPLC-MS machine. During the first analysis farmer A had 241.31ppb albendazole and this was above 100ppb which is the Codex maximum residual limit for albendazole. On the second analysis done farmers ,A, B, C, D, E, F had 239.8ppb, 118.34ppb, 140.63ppb, 254.13ppb, 252.57ppb and 198.62ppb respectively which were above the 100ppb Codex Standard and other farmers were below 100ppb. T-test at 95% significance level showed that there is no significant difference in antibiotic concentration among Dairy farmers that supply milk to Dairy processing companies around Gweru. The effect of albendazole on fermentation of milk was done using back fermentation with 40ml of fermented milk in 500ml of fresh milk. Albendazole was added at different concentrations which were 0, 100, 500, 750, 1000 and 2000 $\mu$ L. pH was measured using a pH meter and lactic acid content was determined by using freshly prepared 0.1N sodium hydroxide. Albendazole slows down the rate of fermentation as pH and percentage lactic acid content was less in the sample with 2000 $\mu$ l albendazole added. pH change was highest in the samples with no antibiotic added. One way ANOVA done at 99% significance level showed that the lactic acid content vary significantly with antibiotic concentration. Regression analysis showed that there is a strong positive correlation ( $R^2 = 0.967$ ) between antibiotic concentration and percentage lactic acid.

## DECLARATION

I, Ruth Nyoka hereby declare that I am the sole author of this dissertation. I authorize the Midlands State University to lend this dissertation to other institutions or individuals for the purpose of scholarly research.

Signature\_\_\_\_\_Date\_\_\_\_\_

## APPROVAL

This thesis entitled “**Occurrence of antibiotic residues in milk, A case study of Gweru**” by Ruth Nyoka meets the regulations governing the award of the degree of **Master of Science in Food Science and Nutrition** of the Midlands State University, and is approved for its contribution to knowledge and literal presentation.

Supervisor .....

Date .....

## **DEDICATION**

I dedicate this piece of work to my late father, Mr. J. R Nyoka. May your soul rest in eternal peace. I also dedicate this to my husband and kids with love.

## **ACKNOWLEDGEMENTS**

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## CHAPTER 1

### 1.1 INTRODUCTION

Food safety is the assurance that food will not cause harm to the consumer when it is prepared and/or eaten according to its intended use (WHO 1998). Consumers want to be confident of what they eat thus it is important to keep food safe and free from contaminants. Contaminants could be physical, biological or chemical. Physical contaminants are those that can be seen by the naked eye and include minerals such as soil, stones, grease, glass fragments. Chemical contaminants include drugs to include antibiotics and other drug residues given to animals, hormones, growth promoters and other chemical residues obtained from food consumed. Microbial contaminants include micro-organisms such as *Salmonella* *ssp*, fungus, *E.coli* and the by-products of their metabolism.

Milk and its products can provide many nutritional benefits to humans; however raw milk can harbour various pathogens and can pose a serious health risk to humans, (Frazer and Westhoff, 2003). To ensure that milk and milk products are safe for consumption all process, preparation and handling procedures should be properly monitored and specifications and guidelines should be adhered to. The nutritional components of milk that make it an important part of the human diet can also support growth of pathogenic microbes. Raw milk carry pathogens such as *Salmonella*, *E. coli*, *Listeria* and so many others which are responsible for causing food borne illnesses, (Murphy and Boor 2000). Besides the harmful effects of the pathogens to humans, the pathogens also cause a condition called mastitis in lactating cows which is the inflammation of the udder (Hogan, 2005). Milk with mastitis should not enter the food chain as it harbours a lot of diseases causing pathogens for example *Salmonella*, *E. coli*, *S. aureus* thus mastitis should be treated with various antibiotics. Milk with mastitis also affects the generally quality and shelf life of milk and its products because

the more microorganisms present cause the degradation of milk components, (Sharma et al, 2011)

Mastitis can be caused by contagious microbes such as *S.aureus*, *S. agalactea* or by environmental pathogens such as *E. coli* and it results in an increase in somatic cell count, increased bacterial count as well as reduced milk quality. Somatic cells are cells from the cow (predominantly white blood cells, otherwise known as leukocytes) that are normally present in milk (Huber 2006). During most mastitis infections, the number of somatic cells present in the udder increases to help the cow fight the infection. There are several types of somatic cells that have different functions in fighting infection. Somatic cells can contain lipolytic and proteolytic enzymes, which degrade fats and proteins in milk, respectively (Ghindinni et al 2002). An increase in somatic cells count during a mastitis infection increases the amount of destructive enzymes present in the milk, which increases the rate of deterioration of the milk fat and protein (Ekuttan et al2007).

In order to deal with mastitis, antibiotics are used worldwide to control bacterial infections and to control the health of farm animals. The antibiotics used in veterinary medicine belong to six major groups which are amino glycosides e.g. gentamicin, Penicillin and cephalosporin ( $\beta$ -Lactam) e.g. cloxacillin, macrolides-e.g. erythromycin, quinolones and fluroquinolones, sulphonamides e.g. trimethoprim and tetracyclines e.g. tetracycline, (Murphy and Boor, 2000). These antibiotics perform various functions in animals when they are administered to treat and prevent certain infections. The functions of antibiotics in animals include as growth promoters, to treat and prevent infections, (Chassagne et al, 2005). Penicillin G was one of the most commonly used antibiotic in the past and penicillin G still has value, particularly in treating streptococcal infections, (Booth, 1998). The emergence of penicillin resistant strains, the finding of new penicillin G insensitive causal agents of mastitis and the continual search

for, and introduction of new therapeutic agents has resulted in the use of a large number of antibiotics from varying groups, (Booth, 1998). Penicillin G, a member of the  $\beta$ -lactam group of antibiotics is rapidly inactivated by  $\beta$ -lactamase, an enzyme produced by *Staphylococci* spp and other bacteria, (Fallah, 2010). However,  $\beta$ -Lactams can be chemically modified to produce semi-synthetic penicillins that are resistant to most  $\beta$ - lactamases for example cloxacillin (Williams, Phillips and Jolly, 2004). The other two major groups which are used in the dairy industry are the quinolones and the sulphonamides (Booth, 1998).

The level and duration of antibiotic diffusion into milk depends upon several factors including the particular antibiotic, its concentration and method of preparation that is whether it is prepared as an aqueous solution or as a suspending medium, (Fallah , 2010). The method of processing markedly influences retention and can affect adhesion of the antibiotic to equipment and pipelines (Teuber and Perreteren, 2000). The amount of antibiotic excreted into milk may vary from eight to eighty percent; usually it averages about fifty percent of the dose administered (Murphy and Boor, 2000). It is therefore difficult to know the exact quantity of antibiotic residue in milk at different milking times after treatment (Riedeker et al 2004). Generally, the concentration of antibiotic in milk decreases rapidly with successive milking, usually at an exponential rate (Ramirez et al, 2003).

There is an increase in the use of antibiotics to treat various infections and to increase profitability among farmers as some antibiotics are used as growth promoters and as therapeutic feeds, (Fallah, 2010). Dairy companies in Zimbabwe mainly rely on just the detection of various groups of antibiotics. Dairy companies also do not detect the levels of antibiotics in milk they only detect the presence of them. Antibiotic residues pose a serious health threat as their consumption may cause antibiotic resistance in humans.



## **1.2 STATEMENT OF THE PROBLEM**

The presence of antibiotics in raw milk is undesirable and raises health and safety concerns for consumers, (Booth, 1998). However current detection methods for antibiotics in milk in Zimbabwe at Dairy companies do not take into account all the potential antibiotics used at the farm. The major antibiotic groups detected in Dairy companies are the  $\beta$ -Lactams, the quinolones and the sulphonamides. Usually when dairy companies detect the presence of antibiotics they use the milk for non-fermented products and the milk still has antibiotic residues. Antibiotics also compromise the quality of fermented dairy products as they interfere with the growth of starter cultures used for making cheese and yogurt. The major detection methods for antibiotics do not quantify all the antibiotics in milk. A continued low level intake of antibiotics from food could result in the build up of antibiotic resistant organisms in humans who are not allergic to the drug, (Teuber and Pereteren, 2000).

## **1.3 SIGNIFICANCE OF THE STUDY**

The study will indicate the levels of antibiotic residues to see if they are within the stipulations set by various regulations. This may enlighten Dairy manufacturer on the importance of better equipment that can detect and quantify antibiotics so that they may not contribute to the antibiotic resistance in humans.

Consumers will also make informed choices on the consumption of both raw milk and various dairy products. Some consumers are not aware of the effects of the antibiotic residues thus this research will enlighten them.

Some farmers are also not aware of the effects of the presence of antibiotic residues in milk thus this research may enlighten them on the importance if proper use of antibiotics and the

importance of adhering to specific periods after the administration of antibiotics in lactating cows.

The researcher will also benefit from the research. From the knowledge gained on the levels of antibiotic residues and the effects of various antibiotic residues in humans as each antibiotic has different effect in humans. The researcher will gain knowledge on the antibiotics and their use. Practicals run using HPLC the researcher will also benefit from the laboratory work to be done.

The institution will benefit as the research will be used by various students to enlighten them on the use and effects of various antibiotics in cattle that is lactating cows.

## **1.4 OBJECTIVES OF THE STUDY**

### **1.4.1 MAIN OBJECTIVE**

To determine the occurrence of antibiotic residues in milk among Dairy farmers in Gweru.

### **1.4.2 SPECIFIC OBJECTIVES**

The research also seeks to achieve the following specific objectives:

- To determine the presence of various antibiotic residues in milk supplied by various groups of farmers around Gweru
- To determine the levels of antibiotic residues in milk.
- To determine the effect of antibiotic treatment on fermented milk.

## **1.5 HYPOTHESIS**

H<sub>0</sub>1: There will be no significant difference on the levels of antibiotics in milk supplied by various farmers.

H<sub>0</sub>2: There will be no significant differences in lactic acid content in milk treated with various antibiotic concentrations.

### **1.6 Scope and Delimitations of the study**

The research is an analytical research. The research analyzed the occurrence of antibiotic residues in milk among Dairy Farmers. Analytical instruments used in the research were the HPLC coupled to a Mass Spectroscopy detector.

The research was carried out in Gweru for the period January to April 2014.

Sample collection of raw milk was done upon delivery at Dairy processing company.

The determination of antibiotic residues in milk samples was done at Central Veterinary Laboratory in Harare.

### **1.7 Limitations and assumptions**

Limited financial resources

The standards which were used were quite expensive. Also money was needed to pay for the laboratory experiments such that instead of doing practicals for three times the researcher eventually analyzed twice.

The following were the assumptions of the study:

- All farmers know about antibiotics and their uses.
- Milk undergo various analyses before receive.
- There are strict regulations and monitoring of milk and milk products in Zimbabwe.

## **1.8 DEFINITION OF TERMS**

**Milk**-is the normal mammary secretion of milking animal obtained from one or more milkings without either addition or removal from it anything, intended for consumption as liquid milk or for further processing.

**Mastitis**- infection of the mammary gland with either environmental or contagious microorganisms.

**Clinical mastitis**- udder infection that shows visible signs for example a swollen udder.

**Subclinical mastitis** = no visible signs of infection of the udder.

**Somatic cells**- these are cells from the animal body. They include leukocytes which are white blood cells which fight against infections such as mastitis, intramammary epithelial cells from the udder secretory tissue.

**Somatic cell count**- the total number of cells per millilitre in milk. The somatic cell counts vary with the state of the animal in milk that is whether it is sick or not.

**HPLC** (high performance liquid chromatography) - a procedure that is used to detect and quantitate compounds in this research to separate, identify and quantitate antibiotics in milk. It consists of a liquid stationary phase and a solid stationary phase. It may also consist of both liquid in the stationary and mobile phases.

## **1.9 Chapter summary**

This chapter introduced the research and explained a brief background on antibiotics. The statement of the problem was highlighted as well as the hypothesis. There is a need to detect and quantitate antibiotic residues in milk as they cause antibiotic resistance, they affect starter culture activity as well as causing allergies.

## **CHAPTER 2**

In this chapter relevant literature is reviewed. What various authorities say about the presence of antibiotic residues in milk is also highlighted. The various groups of antibiotics and their maximum residual limits as per Codex Alimentarius Commission are also indicated. Various method of detecting antibiotic residues in milk are also highlighted. Related issues to the occurrence of antibiotics such as mastitis, somatic cell count are also highlighted.

### **2.1 MILK**

Milk is a rich source of nutrients and has a high water activity, thus it is an excellent media for growth of microorganisms (Abbassi et al, 2011). In raw milk, microorganisms come from inside the udder, animal body surfaces, feed, air, water and equipment used for milking and storage (Frazier and Westhoff, 2003). Milk may also contain organisms from development of mastitis in lactating cows which should be treated using antibiotics in lactating animals. Organisms that grow well in milk include *coliform* species, *Bacillus*, *Staphilococcus* and others.

### **2.2 ANTIBIOTIC RESIDUES IN MILK**

#### **ANTIBIOTICS**

Antibiotics are vital medicines considered as the ultimate strategy for treating both animal and human infections. The effectiveness of antibiotics is however threatened by extensive and inappropriate use of them in various fields such as medicine and agriculture, (Devendra, 2001). In veterinary practice, antibiotics are utilized at therapeutic level primarily to treat diseases and to prevent infection, (Dreyfuss and Faws, 2003). They are also used at sub therapeutic level to increases feed efficiency, to promote growth and to prevent diseases. Presence of antibiotics in foods is caused by either therapeutic treatments or by antibiotic-

supplemented food given to certain animals and the consequences of the presence of antibiotic residues is as numerous to human health as they are to certain processing operations (Botsoglou and Fletouris, 2001). Antibiotics are extensively used worldwide for treating clinical mastitis (CM) and Somatic Cell Count, implying an increased risk of residues in milk and of the development of antibiotic resistance, which is considered to be a major public health threat (Hogan, 2005). The frequent use of antibiotics may result in drug residues that can be found at different concentration levels in food products of animal origin and in milk. Presence of antibiotic residues in food above the maximum level recognized worldwide by various public authorities is illegal (Shammsipur, 2002).

Approximately five to ten percent of the total population is hypersensitivity to penicillin at a concentration as low as one part per billion or people suffer allergic reactions such as skin rashes, hives, asthma, anaphylactic shock (William, Phillips and Jolly, 2004). Where human health is concerned, a number of dangers such as allergic effects and possibilities of microbial selections and of mutations that essentially have two consequences which are the selection of resistant strains and disequilibrium of the normal flora of the digestive tract occur due to the presence of antibiotic residues in foods (Barckrci, 2007).

Antibiotics may also interfere with the manufacture of several dairy products. Concentrations of one part per billion penicillin may delay starter activity during butter production and yoghurt making (Ramirez et al, 2003). Antibiotics decrease the acid and flavor production associated with butter manufacture and they reduce curdling of milk and cause improper ripening of cheese (Topel, 2004).

### 2.2.1 VARIOUS METHODS OF DETECTING ANTIBIOTIC RESIDUES IN MILK

4 different methods are currently available to detect antibiotics.

- **Microbiological tests**- used in detecting antibiotic presence with a bacterial strain which is inhibited or not by the presence of an antibiotic (Kaya and Filaz, 2010). These tests detect numerous antibiotics at thresholds generally very close to the MRL. They are quality tests (presence or absence) but with a known threshold.

Microbiological tests, like certain rapid tests, can be performed by non professionals for example farmers, truck drivers, whilst the other tests can only be performed in adapted laboratories with specialized technicians. Microbiological are rapid tests and are cost effective, that is they are cheap, whereas the other tests are expensive. Microbiological tests were developed many years ago to guarantee milk suitable for processing, with no inhibitors likely to disrupt the lactic fermentations (Gustavson et al, 2003). These microbiological tests are very easy to use and are massively used at different levels that are in laboratories analyzing producers' milk, in dairy industrial laboratories and even on farms.

These tests show the following characteristics (Madigan et al, 2000):

- Variable sensitivity from one molecule to another: this sensitivity (detection threshold) also depends on the sensitivity of the chosen strain to the different antibiotics used.
- Possibility of “additive” effects: it is possible the simultaneous presence of different antibiotics lead to an additive (and even synergic) effect on the tested bacterium.

Detection threshold means the concentration from which a measuring technique shows the unquestionable presence of a molecule (WHO/FAO Conference, 1998). Quantification threshold means the minimum measurable concentration; often higher than the detection threshold

- **Rapid tests** use different technologies. They evidence within a few minutes, but without

quantifying the presence of an antibiotic family. The most often used tests are those in search of  $\beta$ -lactam-antibiotics, but similar tests for tetracycline or other families exist (Kay and Filaz, 2010). These tests are defined more by their functionalities than by the various technologies they employ. These tests provide in a few minutes quality analysis results (presence or absence), generally for an antibiotic family. These tests are used before all other tests in milk where collect trucks are tested before being unloaded. A negative result allows unloading. A positive result generally leads to blocking the truck and performing new tests. These tests are only used for searching  $\beta$ -lactam antibiotics, mainly for two reasons. The reasons are that these antibiotics are the ones most often used on dairy farms and they can contaminate very large volumes of milk. Among the most often used rapid tests for detecting  $\beta$ -lactam-antibiotics are:

- Penzym: enzymatic and colorimetric test. An enzyme (DD Carboxypeptidase) is inhibited by the presence of  $\beta$ -lactam antibiotics.
- Delvo-X-PressM BL: immune-enzymatic test based on the dosage by colorimetry of the excess of a given reagent. In the presence of  $\beta$ -lactam-antibiotics, a “betalactam-tracer” is formed (Ibtsam et al, 2009). Consequently, the quantity of free tracer, able to settle on the reaction tube’s walls decreases.
- Bêtastar: test based on the use of a specific receptor related to gold particles. These receptors migrate differently on a strip depending on whether they are linked to  $\beta$ -lactam-antibiotics or not (Shammary,2000).
- Snap: uses an immuno-enzymatic method. Receptors can link either to the antibiotic (if it is present) or to the antibiotics fixed to the test surface.
- Charm II: an analyzer uses an immunocompetition reaction between the searched molecule and a molecule marked with C14 or H3(Madigan et al,2000). It is a competition test measured by radioactivity.



• **Specific tests**, these tests enables search for a given molecule and quantify it. It is possible to search at the same time for several molecules previously defined. These tests developed in the 1980s due to the advances of electronics and information technology, allowing the development of more and more performant High Performance Liquid Chromatography (HPLC), UV-Visible detection system, and fluorimetric systems (Gustavvson et al, 2003). These methods have very low detection limits, the results obtained enable to settle on the sample's conformity to the Maximum Residual Limit. The limit of these methods is their spectrum as many methods are needed as there are antibiotic families. Certain antibiotics are difficult to identify due to their inability to absorb within the UV. Coupling liquid chromatography (LC) and mass spectrometry (MS) combines the separation power of the liquid chromatography and the structural information on the analysis given by the spectrometer (Gustavvson et al, 2003). New techniques also allowed to dose molecules that do not absorb the UV such as erythromycin using for example a thermospray. The liquid chromatography coupling with mass detector was improved in the middle of the 1990s. Advantages of these techniques LC/MS and LC/MS/MS justify their use for routine analyses in official veterinary drug residue control laboratories (Kaya and Filaz 2010).

• **Identification and quantification tests** are also available. They are, for example, based on the LC-MS-MS principle (Liquid Chromatography and Mass Spectrometry). They are often used as confirmation after rapid screening tests.

### 2.2.2 TYPES OF ANTIBIOTICS

- Dry cow antibiotics which are used at drying off and are oil-based/high antibiotic concentration and these last for approximately 56 days (Ding and Mou, 2000).
- Milking cow antibiotics which are used during lactation and are water based/low antibiotic concentration. These last for approximately 3 days (Ding and Mou, 2000).

### 2.2.3 GROUPS OF ANTIBIOTICS

#### BETA-LACTAM ANTIBIOTICS

The  $\beta$ -Lactams are the oldest and mostly used antibiotics among all others (Ghinidi, et al 2002).  $\beta$ -lactam group of antibiotics are used especially to fight mastitis which is a serious disease that causes considerable economic losses in world's industry (Riediker, et al. 2004).

Penicillins and cephalosporins have both  $\beta$ - lactam rings where in the case of penicillins it is fused to a five-membered thiazolidine ring, and in the case of cephalosporins, it is fused to a six-membered  $\Delta^3$ -dihydrothiazine ring as explained by Fagerquist and Lightfield (2003).

$\beta$ -lactam ring in antibiotics of this group makes them chemically reactive with the instability of its carbonyl group towards nucleophilic attack. Bacteria produce enzymes that catalyze the hydrolysis of the  $\beta$ -lactam ring to defend against most penicillins by deactivating them.

Cephalosporins are less sensitive to catalytic degradation however penicillinases are classified as penicillinase-resistant or 'penase' resistant (Fagerquist and Lightfield 2003).

Classes of  $\beta$ - lactams with bulky side chains are attached to the 6-amino penicillanic acid (6-APA) or 7-amino cephalosporinic acid nuclei, respectively. Penicillins are used extensively because of their antibacterial activity against both gram-positive and gram-negative organisms (Devendra, 2001). Penicillins produce different degradation products especially in organic solvents because of its limited stability (Shammsipur, et al. 2002). Although other antibiotics and chemotherapeutic are available to cure infections in lactating cows, the major problems encountered by the dairy industry are caused by penicillin (Gustavsson, et al. 2002). Penicillins are not very toxic but in sensitized individuals it causes strong allergic reactions. Ampicillin, a semi-synthetic penicillin-like drug is also widely used.

Determinations of ampicillin have critical importance since the presence of degradation and PhG and 6-APA may decrease activity of ampicillin and cause some side effects and allergic reactions in human body as explained by (Shammsipur, et al. 2002). Antimicrobial activity caused by ampicillin can be extended to include gram-negative bacteria such as *Haemophilis influenza*, *Escherichia coli* and *Proteu mirabilis* and as high as 10% of over sensitive reactions are observed using this group of antibiotics (Fernandes et al, 2007).

## **SULPHONAMIDES**

The sulphonamide drugs that are used in animal production are soluble in polar solvents such as ethanol, acetone, acetonitrile and chloroform but insoluble in nonpolar solvents (Shammsipur et al, 2003). This group has wide variety of polarity with amphoteric properties due to the basic character of the para-NH<sub>2</sub> group and due to N-H linkage adjacent to the sulphonyl group (Eukutan et al 2007). P-aminobenzenesulphone moiety is a part of many sulphonamides which reveals antimicrobial activity. Sulphonamides have been benefited as antibiotic agents in veterinary practice for several decades and are the fifth most widely used group in veterinary antibiotics in European Union countries, accounting to 2% of sales in 1997 ( Ramirez et al, 2003). The antimicrobial activity is exhibited by tri-methoprim that is why they are frequently co-administered with this compound. Among many sulphonamides that have been defined, only few are approved for animals as veterinary medicine (Hogan, 2005).

## **TETRACYCLINES**

The Tetracyclines including Tetracycline, Oxytetracycline and Chlortetracycline are broad-spectrum antibiotics widely used in animal husbandry for both prevention and treatment of diseases and feed additives to promote growth (Shammsipur et al, 2002). The antibiotic residues in food could influence the bacterial composition of the intestinal

microflora, their metabolic activity and the metabolism of endogenous compounds.

Tetracyclines in milk may cause staining of teeth in young children. WHO and FAO have set standards for acceptable daily intake (ADI) and maximum residue levels (MRLs) in foods, in order to protect humans from harmful effects of drug residues in milk. WHO, European Union (EU) and Chinese ministry of agriculture have established a MRL of 100ng/g for Tetracycline, Oxytetracycline and Chlortetracycline. According to Ghinidi et al (2002), the U.S. food and drug administration (FDA) has set the MRL of 300ng/g for total residues of tetracyclines. The acceptable MRL for tetracyclines (singly or in combination) as recommended by the Joint FAO/WHO Expert Committee on Food Additives is 100ng/g for bovine milk. Studies conducted in Kenya showed samples of milk to contain tetracyclines at levels exceeding the established maximum residual limit. (Shammispur et al, 2002). Studies in Kuwait showed that 18% of milk samples had tetracyclines residues above the permitted limit (Ghinidi et al, 2002). In Czech Republic, studies showed presence of tetracycline residues in all and oxytetracycline residues in 50.6% of analyzed raw cow milk samples (Mehran et al, 2011).

## **BENZIMIDAZOLES**

These are a chemical class of compounds with broad anthelmintic activity that are widely used in livestock and other domestic animals including plants as well (Madigan et al, 2000). They are used to control parasitic worms. They are also used in human medicine. Benzimidazole anthelmintic drugs are widely used in veterinary medicine for the treatment of helminth infections in food-producing animals (Kaya and Filaz, 2010). These infections result in reductions in milk yields and weight gain. In the EU, 11 benzimidazoles and pro-

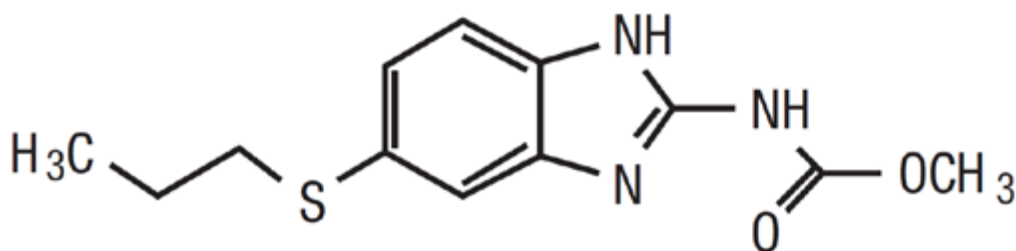
benzimidazoles are approved for treatment of food-producing animals giving rise to 20 potential residues. The main concerns over the presence of benzimidazole residues in milk are related to their teratogenic and embryotoxic properties (Wilson et al, 1998)

All benzimidazoles are poorly soluble in water. For these reason, most liquid formulations for cattle, sheep and goats are liquid suspensions for oral administration or intraruminal injection. Numerous feed additives are also used together with benzimidazoles, especially for pig and poultry, but also for ruminants. Some but few benzimidazoles are also available as injectables or as slow to release boluses. The molecular mode of action of all benzimidazoles consists in binding to tubulin, a structural protein of microtubules (Wilson et al, 1998). These microtubules are important organelles involved in the motility, the division and the secretion processes of cells in all living organisms. In the worms the blocking of microtubules disturbs the uptake of glucose, which eventually empties the glycogen reserves. This blocks the whole energy management mechanism of the worms that are paralyzed and die or are expelled. Since cell division is also disturbed worm egg production and development is also blocked by benzimidazoles, thus they also have an ovicidal effect (WHO/FAO Conference, 1998).

### **Safety of benzimidazoles**

Benzimidazoles have a much higher affinity for tubulin in the worm cells than for the tubulin in the cells of livestock or pets (Shamary, 2000). This makes it possible that at the therapeutic dose the drug kills the nematodes without harming the host. As a general rule, livestock and pets tolerate benzimidazoles very well. The safety index ranges from 3 to 20, mostly greater than 10 (Wilson et al, 1998). Benzimidazoles do not bind to host tissues and are rapidly excreted. This allows withholding periods for meat between 1 and 4 weeks, depending on the compound, the formulation and the administered dose. Use on dairy livestock is often not allowed in many countries, but this also depends on the specific product and its usage.

Albendazole, (as well as netobimin and ricobendazoole), cambendazole and parbendazole can be teratogenic, that is they can cause malformations in the embryos, and it is recommended not to use them in pregnant livestock. Without reliable use instructions they can be easily overdosed, and they may contain ingredients that are toxic for animals.



*Abendazole which is the main active ingredient for benzimidazoles, adapted from Gil-Grande 1993.*

### 2.3 Categories of Antibiotic Use in Dairy production

As **growth promoters**-antibiotic are used to improve performance on growth. They may produce growth because of thinning of the mucus membrane of the gut, facilitating better absorption, altering gut mortality to enhance better assimilation, producing favorable conditions of beneficial microbes in the gut of the animals by destroying harmful bacteria and partitioning proteins to muscles maceration .(Nicholas et al, 2004). Antibiotics also favor growth by decreasing the degree of activity of the immune system, reduced waste of nutrients and reduce toxin formation. (Nisha, 2008). In most cases only young growing animals are responsive to antibiotic meditated growth. Older heifers may receive concentrate mixes to improve feed efficiency and growth.

Antibiotic use in therapeutics- antibiotics can be used in cases of various diseases and conditions such as pyrexia, wounds and viral diseases. They should be used because of the role of microbiological agents is to kill the rapidly evading cells. The most common disease

control use of antibiotics in dairy is intramammary dry cow treatment as described in the mastitis discussion. Dry cow antibiotic treatment is an integral part of a good mastitis management and milk quality program.

Antibiotic use in prophylaxis- they are used to prevent possible infections. (Nisha, 2008). The antibiotics are however specific to their spectrum of activity only in the active multiplication stage of bacteria. (Nicholas et al, 2004). Milk replacer typically contains an antibiotic for prevention of gastroenteritis. A combination of ox tetracycline and neomycin are commonly used, while chlortetracycline, decoquinatate and lasalocid are available for use in milk replacers as well. Coccidiostats, such as decoquinatate, lasalocid, and monensin, may be used to prevent coccidiosis in young calves or heifers on farms with disease problems. Other uses of antibiotics include during production and during processing of milk and milk products where antibiotics are used to destroy evading microorganisms during transportation and storage as well as during processing, (Nisha, 2008)

Pathological effects produced by antibiotic residues in food

These include the following:

- Transfer of antibiotic resistant bacteria to humans
- Immunopathological effects
- Autoimmunity
- Carcinogenicity(sulphamethizine, oxytetracycline, mutagenicity)
- Hepatotoxicity
- Reproductive disorders
- Bone marrow toxicity
- Allergic reactions(penicillin)

## **2.4 SOMATIC CELLS**

Somatic cells are simply animal body cells present at low levels in normal milk (Ekuttan et al, 2007). High levels of these cells in milk cause reduced-quality that is caused by an intramammary bacterial infection (mastitis). The majority of the cells in a somatic cell count are leukocytes (white blood cells), and some are cells from the udder secretory tissue (epithelial cells) (Ghidini et al, 2002). Epithelial cells are part of the normal body function and are shed and renewed in normal body processes. White blood cells serve as a defence mechanism to fight disease (infection), and assist in repairing damaged tissue (Mehran et al, 2011).

Somatic cell count (SCC) is the total number of cells per milliliter in milk and SCC is composed of leukocytes, or white blood cells, that are produced by the cow's immune system to fight an inflammation in the mammary gland, or mastitis (Abbassi et al, 2011). Since leukocytes in the udder increase as the inflammation worsens, SCC provides an indication of the degree of mastitis in an individual cow or in the herd if bulk tank milk is monitored (Bishop and White, 1984). Increase in SCC in milk, causes a decrease in the processibility of milk as well the keeping quality. This is due to increase in the loss of casein and milk fat, reduced curd firmness due to high SCC. High somatic cell count affect quality of pasteurized milk and SCC cause a decrease in shelf life, increased casein breakdown, decrease cheese yield, cause the development of off-flavour at a faster rate in milk(Devendra,2001).

### **Causes of a High Somatic Cell Count**

High Somatic Cell Count levels are abnormal and undesirable and somatic cell count could be due to pathogens which could be environmental or contagious, infection of the mammary gland, it could be due to the stage of lactation and cow's age, stress and season, udder injury



and indirect causes such as poor milking procedures, faulty milking equipment due to poor installation and other causes as explained below.

The major factor that causes elevated Somatic Cell Count is an infection of the mammary gland, (William, Phillips and Jolly, 2004). An increased bulk tank somatic cell count is related to increased herd infection prevalence and decreased milk production. The normal Somatic Cell Count in milk is generally below 200,000 per ml, but may be below 100,000 in first lactation animals or in well-managed herds (Murphy and Boor, 2000). A Somatic Cell Count above 250,000 - 300,000 per ml is considered abnormal and is also an indication of bacterial infection causing inflammation of the udder (Murphy and Boor, 2000).

The most common organisms that infect the milk-producing gland are classified into two groups viz contagious pathogens and environmental pathogens. The contagious pathogens (*Staphylococcus aureus*, *Streptococcus agalactiae*, and some others) generally cause the greatest Somatic Cell Count increase. An infection by an environmental pathogen (*Streptococcus*, *Corynebacterium bovis*, and coagulase negative Staphylococcus) usually causes considerably less Somatic Cell Count elevation (Chen et al, 2001). However, Somatic Cell Count range between individual infected cows can be quite broad.

**Cow age and stage of lactation**-General observation implies that Somatic Cell Count increases with advancing age and stage of lactation. Eberhart and co-workers at The Pennsylvania State University (2006) separated cows into groups by infection status. They found little obvious change in SCC either in late lactation or as a cow ages in uninfected cows that is if SCC is high an infection exists in the mammary gland. Elevated SCC may occur in milk in late gestation and for a few weeks following calving, regardless of infection status (Huber, 2006). This SCC elevation appears to be part of a cow's natural immune

system response in preparation for calving, to enhance the mammary gland defence mechanisms at this critical parturition time (Kamkar et al, 2011). Quarters with no infections generally have a rapid decline in SCC within a few weeks postpartum.

**Stress and season-**Stresses of various types and oestrus (heat cycles) have been implicated in causing increases in SCC (Fallah, 2010). However, research attempts to experimentally induce SCC changes in uninfected cows have shown only modest or no effects on milk SCC. SCC levels usually are lowest in a clean, dry, comfortable environment (Mehran et al, 2011). Weather and management factors play an important part relative to the control of mastitis. Mastitis control principles must be maintained at all times. The increased incidence of clinical mastitis (udder infection that shows visible signs) in the summer months is generally due to a warm and moist environment that increases pathogen exposure and numbers (Dreyfuss and Faws, 2003). Animals are usually stressed due to high temperatures and excess humidity and this may increase susceptibility to new infection and thus, higher SCC.

**Udder injury-**Tissue damage from injury in the individual cow may temporarily elevate SCC even without infection (Verma, 2004). Such instances usually would be of short duration and improve as healing occurs. Damaged tissue is quite susceptible to infection so prevention of injury is important and also to eliminate ledges, debris, slick floors.

**Indirect causes (Devendra, 2001)-**Poor milking procedures contribute heavily to new rate of infection due to transmission of the disease at milking time. The result is an elevated SCC. Faulty milking equipment due to poor installation or maintenance can cause tissue trauma, teat damage, poor milk-out, erratic vacuum levels, and also can transmit infectious agents at milking time. A complete system analysis should be conducted by competent equipment personnel at least twice a year (every six months) or after every 1,000 hours of system operation to minimize such dangers (Fallah 2010). Extraneous voltage causes cow

apprehension, teat irritation, uneasiness and poor milk-out, and these conditions contribute to new mastitis problems and elevate SCC in most cases.

## 2.5 MASTITIS

Mastitis is an inflammation of the udder, typically caused by a microbiological infection (Teuber and Pereten, 2000). Mastitis can occur in all mammals, including humans. Many types of microbes can cause infection and they can be transmitted from both environmental sources (for example, contaminated water, soil, bedding) and from contagious sources (from other infected cows), (Verma, 2004). The microbes can enter the udder and multiply. The microbes can enter milk as it passes through the udder during the milking process. Mastitis in dairy animals can be a source of disease causing pathogens and spoilage organisms in milk.

The most common organisms that cause mastitis are classified into two major groups which are contagious pathogens and environmental pathogens (Wilson et al, 2003). *Staphylococcus aureus*, *Streptococcus agalactiae* are the contagious pathogens that generally cause the greatest SCC increase. An infection by environmental pathogens (*Strep. dysgalactiae*, *Strep. uberis*, *Corynebacterium bovis* and Coagulase negative Staphylococcus) usually causes considerably less SCC elevation (Riediker et al 2004).

Milk from cows infected with mastitis generally has higher total bacteria counts and somatic cell counts than milk from uninfected cows. Therefore, bacterial counts and somatic cells counts are used by dairy farmers and processors as indicators of milk quality (Ding and Mou, 2000). In general, the higher the bacterial counts, the lower the milk quality. Milk from mastitis cows may have off-flavours and may undergo deterioration of the milk fat and protein more quickly than milk from healthy cows.

There are regulatory standards for microbial numbers (total bacteria count) as well as quality control and human health parameters (somatic cell count, and antibiotic drug residues) in milk, (Grade a Pasteurized Milk Ordinance, 2005). Cows with mastitis are usually separated from the herd to help control the spread of infection and to ensure that good quality milk is produced on that farm (Chen et al, 2001). On some farms, cows with mastitis are treated for the infection with antibiotics. The milk from the treated cows is either discarded or diverted to a separate tank to prevent contamination of milk collected from healthy cows. Milk from cows treated with antibiotics should not be used for human consumption (Huber 2006).

### **2.5.1 REDUCING SOMATIC CELL COUNT AND MASTITIS**

There are two methods of reducing SCC and mastitis. The first method, culling cows, is a short-term solution which can quickly reduce SCC in the bulk tank and the other method, controlling mastitis, is a long-term solution which should be the basis of a sound management program, (Prandin, 2009).

**Culling Cows** -Cows with a very high SCC that do not respond to antibiotic therapy or that have chronic mastitis may have to be culled from the herd. Cows with high SCC have mastitis that is caused by contagious bacteria, primarily *Staphylococcus aureus* but also *Streptococcus agalactiae* (Murphy and Boor, 2000). The most common source of contagious bacteria is other infected cows; whereas, environmental pathogens are most commonly isolated from recently calved and dry cows (Kamkar et al, 2011). These bacteria, especially *Staphylococcus aureus*, often do not respond to routine antibiotics and should be cultured to determine a more appropriate antibiotic for them. If the cow then does not decrease in SCC after more extensive treatment, it should be culled.

**Prevention through Nutrition** is one aspect of a sound management program. Increasing a cow's resistance to mastitis pathogens on the teat-end is an important component of immunity in the dairy cow (Khush et al, 2010). Nutrition helps in maintaining immunity. Inadequate energy or deficiencies of nutrients affect resistance of cows to infection. Diets may be deficient in nutrients that are related to immune competence. A balanced ration with proper amounts of minerals and vitamins improves the ability of a cow to ward off bacterial challenges (Botsoglou and Fletouris, 2001). Selenium and vitamin E are related to healthy tissue in the mammary gland thus these nutrients are among other nutrients which are important in Dairy cows.

**Prevention through Sanitation and Management**-Improving sanitation to decrease mastitis is simply keeping the udder clean and free of pathogenic bacteria that cause mastitis (Mehran et al, 2011). Major teat contamination can be avoided by eliminating mud and preventing wading in ponds. Bedding must be dry at all times. Straw and sand beddings are generally recommended, green wood sawdust should be avoided, clean sod or new bedding is essential for all springing heifers and dry cows as well as milk cows (Cinquina et al, 2003). The grass sod in the pasture or dry lot should be free of mud and objects such as sticks that damage the udder. Special care must be exercised with heifer management. Calves should be reared in separate pens, fly population must be controlled to decrease the spread of mastitis-causing bacteria and springing heifers should be separated from cows (Ekuttan et al, 2007).

**Dry Cows and Springing Heifers** –The risk of intra-mammary infections is greatest during the early and late dry period when pathogens are not flushed out on a day-to-day basis (Cinquina et al, 2003). Infection rate in dry cows is directly related to the bacterial population on the teat-end. Protective sealant dips are beneficial for teat protection and should be used after treating dry cows (Bidlingmeyer, 1987). Before sealing teats, extreme care must be

taken to avoid contamination of the teat. Dry cow treatment of all quarters of all cows is recommended, but the largest danger with any intra-mammary infusion is recontamination. The teat-end must be cleaned and sterilized with a pre-dip before insertion of the dry-treatment tube (Chen et al, 2001). Insertion of the tube through a drop of pre-dip to a maximum depth of 3/8 inch reduces bacterial contamination entering the teat canal and minimizes damage to the keratin teat plug at the end of the teat (Devendra, 2001).

Dry cow treatment helps eliminate and prevent new infection during the dry period. During the late dry period, new infections should be guarded against and the springing period is a vulnerable time as dry cow therapy has dissipated and mammary tissue is in a growing phase without antibiotic protection (Polpelka et al, 2004). Colostrum is an ideal medium for growth of pathogenic bacteria. Springing cows may be retreated if needed, but caution must be taken to prevent contamination, (Bishop and White, 1984). Monitoring dry cows for inflammation after treatment is necessary and regenerating udder tissue must be monitored for swelling and inflammation. Prior to calving, dry cow teats may be cleaned and dipped as needed and the seal at the end of the teat should not be broken during the teat cleaning.

**Lactating Cows** -Proper and sanitary milking procedure should be followed with proper equipment. To minimize contamination of milk with drug residue, only legal medication should be used (Ding and Mou, 2000). Cows treated with any medication should be properly marked for milk withholding and withdrawal time required should be followed. Before allowing milk into the tank all treated and fresh cows should be tested.

**Stray Voltage**-Stray voltage is damaging electricity from many sources in milking parlors that can be grounded through a cow (Dreyfuss and Faws, 2003). A small voltage can cause production of epinephrine, which blocks the effect of oxytocin that is required for milk letdown. Normal mechanism of milk letdown allows most of the milk to be removed.

Incomplete removal of milk causes mastitis due to rapid bacterial growth. Frequent milking has been used to wash out pathogenic bacteria. When abnormal bacteria are observed, stray voltage should be checked by an electrician and the power-company representative (Ghindini et al, 2002). Common causes are 120-volt motors, static electricity, off-farm voltage leak and ungrounded motors (Fallah, 2010). New construction should have a grid installed beneath the floor to prevent stray voltage from grounding through the cow. Well-grounded 220-volt motors in all areas where possible to keep both 110 legs of the incoming service balanced and reduce the likelihood of voltage drift should be used. Sheet iron roofing generates more static electricity during windy conditions.

The incidence of high SCC and mastitis are associated with exposure to causative agents as well as the cow's level of resistance. Risk factors associated with high SCC are associated with poor primary management practices including milking equipment and technique, housing, cleanness of the environment, feed hygiene and stress (Fallah, 2010) Secondary factors such as parity, lactation stage, breed, udder conformation and milk production are also important.

## **2.5.2 EFFECT OF HIGH SOMATIC CELL COUNT ON MILK QUALITY AND HUMAN HEALTH**

Subclinical mastitis alters the composition of the milk in addition to suppressing milk yield (Bramley, 1992; Harmon, 1994). There is a direct relationship between SCC and milk quality. An elevated Somatic Cell Count in milk has a negative influence on the quality of raw milk. Subclinical mastitis is always related to low milk production (Bramley, 1992; Harmon, 1994), changes to milk consistency (density), reduced possibility of adequate milk processing, low protein and high risk for milk hygiene since it may even contain pathogenic organisms. Mastitis or elevated SCC is associated with a decrease in lactose,  $\alpha$ -lactalbumin,

and fat in milk because of reduced synthetic activity in the mammary tissue (Devendra, 2001). The largest negative consequences of the presence of SCC are related to shorter shelf life and less sensory content or undesirable organoleptic characteristics of the final product, due to enzymatic activities of somatic cells (Töpel, 2004). The higher levels of free fatty acids in high cell count milk may produce a rancid flavour. Cheese production from high somatic cell-count milk has been reported to be lower than from low somatic cell-count milk (Bishop and White, 1984). Decreasing SCC from 340,000 to 240,000 cells/ml increase cheese yield by 1% and decreasing SCC from 640,000 to 240,000 cells/ml increased cheese yield by 3.3% (Riediker et al, 2004). The high presence of SCC in milk affects the activity of yogurt fermentation (Tamime and Robinson, 1999), and can even stop this process. Fernandes et al. (2007) studied the effect of SCC in raw milk on the chemical and physical properties of yogurt. He concluded that an increase in SCC causes an increase in fatty acids in yogurt during the preservation period and thus shortens the time of preservation of this product. The reduced heat stability of high SCC milk causes flocculation during heat treatment processes such as pasteurization and evaporation.

## **2.6 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)**

### **2.6.1 PRINCIPLE OF HPLC**

This is separation based on the analyte's relative stability between two liquids that make up the mobile phase and the stationary phase (Sharma (2010). This is separation of compounds dissolved in a solution. The sample should be in liquid form. Sample mixtures are injected into a column and the components in the mixture pass through the column at different rates depending on their partition behaviour between the mobile and the stationary phase (Huber, 2006). The mobile phase must be degassed to eliminate air bubbles. Problems caused by dissolved air in the eluent is that the delivery pump will be unstable and there will be more noise and la

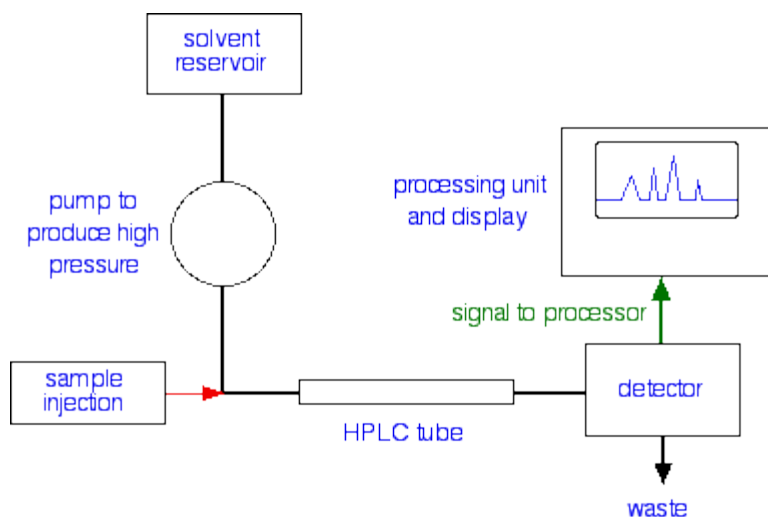


rge baseline drift in detector cell (Hogan, 2005). In order to avoid these problems, the eluent must be degassed. Since the components of the sample have different affinities for the stationary phase, they exit the column at different rates or times (Riediker et al, 2004). The rate of movement determines the time taken by a substance to be eluted and this is referred to as retention time. This time is measured from the time at which the sample is injected to the point at which the display shows a maximum peak height for that compound according to Huber (2002). Different compounds have different retention times. For a particular compound, the retention time will vary depending on: the pressure used (because that affects the flow rate of the solvent), the nature of the stationary phase (not only what material it is made of, but also particle size) the exact composition of the solvent the temperature of the column. The injector places the sample at a high pressure flow in as narrow a column as possible so that the sample enters as a low volume plug and homogeneously (Hogan, 2005). The system could be automated or manual. Advantages of HPLC over other methods of separation are, (Chatwall 2010):

- High separation capacity, enabling the batch analysis of multiple components
- Superior quantitative capability and reproducibility
- Moderate analytical conditions

The advantages of HPLC, the sample does not need to be vaporized, generally high sensitivity, low sample consumption, easy preparative separation and purification of samples.

### 2.6.2 Schematic diagram for HPLC



*Adapted from Sharma (2010)*

*Fig 2.6.2 schematic diagram for HPLC*

**Injection of the sample-**The injector serves to introduce the liquid sample into the flow stream of the mobile phase (Ramirez et al, 2003). Typical sample volumes are 5-to 20-microliters ( $\mu\text{L}$ ). This means very small quantities of the sample are injected and this increases the efficiency of separation. The injector must also be able to withstand the high pressures of the liquid system. An autosampler is the automatic version and it is used when the user has many samples to analyze and the system can be highly automated (Huber, 2006).

**Pump-** The pump forces a liquid (called the mobile phase) through the liquid chromatography at a specific flow rate, expressed in millilitres per min ( $\text{mL}/\text{min}$ ), (Wilson et al, 2003). Normal flow rates in HPLC are in the 1-to 2- $\text{mL}/\text{min}$  range. Typical pumps can reach pressures in the range of 6000-9000 psi. The pump can deliver a constant mobile phase composition (isocratic) or an increasing mobile phase composition (gradient), (Topel, 2004).

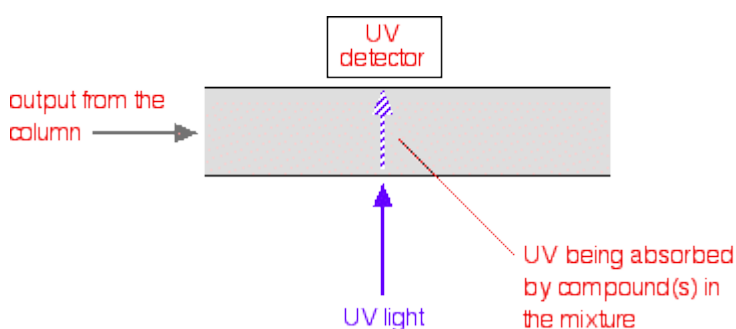
**Column-** the column's stationary phase separates the sample components of interest using various physical and chemical parameters. The small particles inside the column are what

because the high back pressure at normal flow rates. The pump must push hard to move the mobile phase through the column and this resistance causes a high pressure within the

Chromatograph (Fallah, 2010).

Detector: The detector can see (detect) the individual molecules that are eluted from the column. Bidlingmeyer (1987) explained that, a detector serves to measure the amount of those molecules to enable quantitatively analysis of the sample components. The detector provides an output to a recorder or computer those results in the liquid chromatogram (the graph of the detector response).

There are several ways of detecting when a substance has passed through the column. A common method which is easy to explain uses ultra-violet absorption. Many organic compounds absorb UV light of various wavelengths. If you have a beam of UV light shining through the stream of liquid coming out of the column, and a UV detector on the opposite side of the stream, you can get a direct reading of how much of the light is absorbed. The amount of light absorbed will depend on the amount of a particular compound that is passing through the beam at the time.

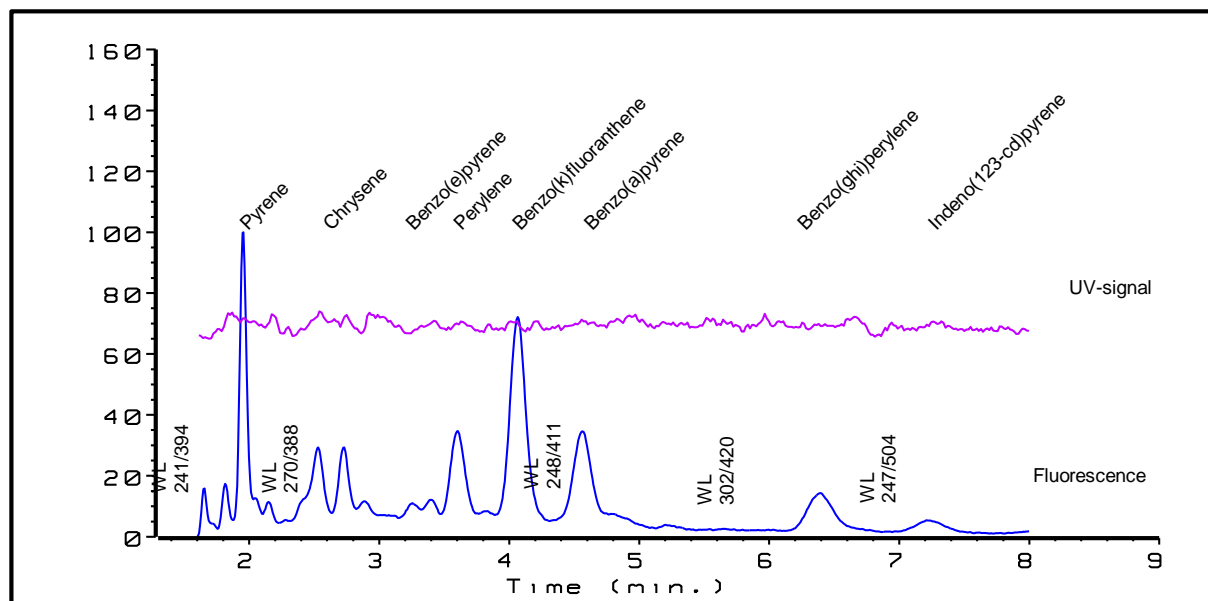


*Adapted from Huber, (2004)*

### 2.6.3 Expected nature of results

The output will be recorded as a series of peaks - each one representing a compound in the

mixture passing through the detector and absorbing UV light if a UV detector is being used. The peaks can be used as a way of measuring the quantities of the compounds present. The area under the peak is proportional to the amount of a substance which has passed the detector and this area can be calculated automatically by the computer linked to the display, (Huber, 2002).



PAH's extracted from soil;  
 Sup.LC-PAH 150x4.6mm;  
 Solv.: H<sub>2</sub>O/CH<sub>3</sub>OH= 10:90

*Adapted from Chatwall (2010)*

*Fig 2.6.3 Nature of results on HPLC Analysis*

The peak and the width of peaks are important. Each peak represents a certain item and standards are used in the identification of the substances. The peak width which is considered under the curve measures the concentration of various substances as explained by Chatwall (2010).

Computer: Frequently called the data system, the computer not only controls all the modules

of the HPLC instrument but it takes the signal from the detector and uses it to determine the time of elution (retention time) of the sample components (qualitative analysis) and the amount of sample (quantitative analysis), (Murphy and Boor, 2000).

#### **2.6.4 Types of HPLC**

***Normal phase HPLC***-The column is filled with tiny silica particles and the solvent is non-polar - hexane, for example. A typical column has an internal diameter of 4.6 mm or it may be less than that and a length of 150 to 250 mm (Nagele and Huber, 2006). Polar compounds in the mixture being passed through the column will stick longer to the polar silica than non-polar compounds will. The non-polar ones will therefore pass more internal diameter of 4.6 mm or less, and a length of 150 to 250 mm.

***Reversed phase HPLC***-In this case, the column size is the same, but the silica is modified to make it non-polar by attaching long hydrocarbon chains to its surface - typically with either 8 or 18 carbon atoms in them. A polar solvent is used - for example, a mixture of water and an alcohol such as methanol. There will be a strong attraction between the polar solvent and polar molecules in the mixture being passed through the column (Prandin, 2009). There won't be as much attraction between the hydrocarbon chains attached to the silica (the stationary phase) and the polar molecules in the solution. Polar molecules in the mixture will therefore spend most of their time moving with the solvent.

Non-polar compounds in the mixture will tend to form attractions with the hydrocarbon groups because of van der Waals dispersion forces (Topel, 2004). These will also be less soluble in the solvent because of the need to break hydrogen bonds as they squeeze in

between the water or methanol molecules, for example. They therefore spend less time in solution in the solvent and this will slow them down on their way through the column (Tamime and Robinson, 1999). That means that now it is the polar molecules that will travel through the column more quickly. Reversed phase HPLC is the most commonly used form of HPLC.

### **2.6.5 Factors affecting HPLC**

- Solvent effect- Water is a “weak” solvent. The use of organic solvents helps to reduce retention time thus elution will be fast, (Huber, 2006). The more organic the solvent, the less the retention time. Organic solvents must be miscible with water.
- pH- Affects ionisable compounds such as organic acids and organic bases. In reverse phase we need to suppress ionization as much as possible thus there should be a very precise pH control (Riediker et al, 2004).
- Sample effect- more components in the sample means there will be more peaks
- Column parameters- column materials, stationery phase, deactivation, coating material
- Instrument parameters- temperature, flow, signal, sample sensitivity, and detector.

Quantitative analysis- Quantitation is performed with peak area or height. Calibration curve created beforehand using a standard is very important in quantitating a particular compound in a sample (Wilson et al, 2003). The calibration curve can then be used by a method called extrapolation to quantitate the substances in the samples (Sharma 2010).

Qualitative analysis- Identification based on retention time. Certain substances have retention times that are known and these can be used in the identification of various compounds (Huber 2006). Acquisition of spectra with detector helps also to identify various compounds.

The detectors which may include UV spectra, MS spectra. Transfer to other analytical instruments after preparative separation can also be done to detect various components of a sample.

### **2.6.6 MS-MS**

The method provides a higher sensitivity and selectivity enabling less extensive sample preparation, greater sensitivity via increased selectivity (Shamispur, et al, 2002). It allows the use of shorter HPLC columns, this enables shorter run times and higher sample through put.

These translate into time and effort saving as well as providing more sensitive method (Bramley, 1992).

### **2.7 Milk pasteurization**

Pasteurisation was named after Lewis Paster, a food microbiologist who discovered that simple heating could destroy spoilage pathogenic organisms in wine (Jay et al, 2005). Pasteurisation simply means the heating of every food particle in milk to a specific temperature and time to reduce undesirable enzymatic activities and bacteria to negligible levels and spoilage pathogenic organisms. It is a function of time and temperature combination. For milk with a high fat content usually higher by ten percent, temperature should also be increased by five degrees (Doyle et al, 2005). Pasteurised milk should have less than 300 000 cfu/ml, it should also have less than three coliforms per ml and should not contain an antibiotic at a level that exceeds 0.01units (Jay et al, 2005). Pasteurised milk should be phosphatase negative and should not contain products that are unsafe for human consumption. Batch pasteurisation temperature are 63 to 65 °C for 30 minutes, some modern

plants use the high temperature short time method (HTST) which use a temperature of 72°C for 15 seconds (Frazier and Westhoff, 2002). Pasteurised milk should be cooled immediately to prevent contamination during a long cooling process.

## **2.8 Dairy starter cultures**

These are carefully selected microorganisms which are deliberately added to milk to initiate or carry out desired fermentation under controlled conditions in the production of fermented milk products, (Ghandhi, 2006). Starter cultures can be used as a single strain, mixed or multiple strains depending on the type of product desired. The ability of a starter culture to perform its function depends on the purity and activity of the starter culture. The functions of starter cultures are as follows, (Doyle et al, 2005):

- Production of primarily lactic acid and a few other organic acids such as formic acid and acetic acid.
- Coagulation of milk and changes in body and texture in the final product
- They also help cheese ripening by their enzyme activities.
- Produce antibacterial substances in the final product and they may also possess functional properties.
- Possess proteolytic and lipolytic activities.

An ideal starter culture should be selected for the preparation of various fermented products from milk with the following properties:

- It should be quick and steady in acid production
- It should produce products with fine and clean lactic acid flavor
- It should not produce any pigment, gas, off-flavors and bitterness in the finished product and it should be associative in nature in product development (Atlas, 1995).



## **Types of starter cultures**

Starter cultures can be classified into two major groups. (Gandhi, 2006). These are that they can be classified basing on physiological and growth characteristics such as mesophilic and thermophilic. They are also classified depending on whether they are heterofermentative or homofermentative.

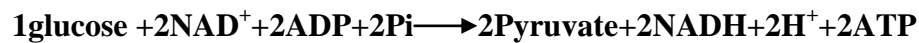
Mesophilic lactic acid starter cultures have an optimum growth temperature of 20 to 30 °C and examples include *Lactococcus* and *Leuconostoc spp.* They are used in cheese production where important characteristics are acid production activities, gas production, and enzymatic activity for cheese ripening, (Lund et al, 2000). Thermophilic lactic acid cultures microorganisms favour optimum temperatures between 37 to 45°C. They are generally employed in yogurt production, acidophilus milk and swiss type cheese. They include species such as *Lactobacillus* and *Streptococcus*. These grow in association with milk to form the typical yogurt starter culture (Doyle et al, 2005). This type of growth is considered symbiotic because the rate of acid development is greater when two cultures or more are grown together. Important metabolic reactions in thermophilic organisms include acid development, flavour compounds development, ropiness and consistency, proteolytic and lipolytic activities and immunity improvement, (Jay et al, 2005).

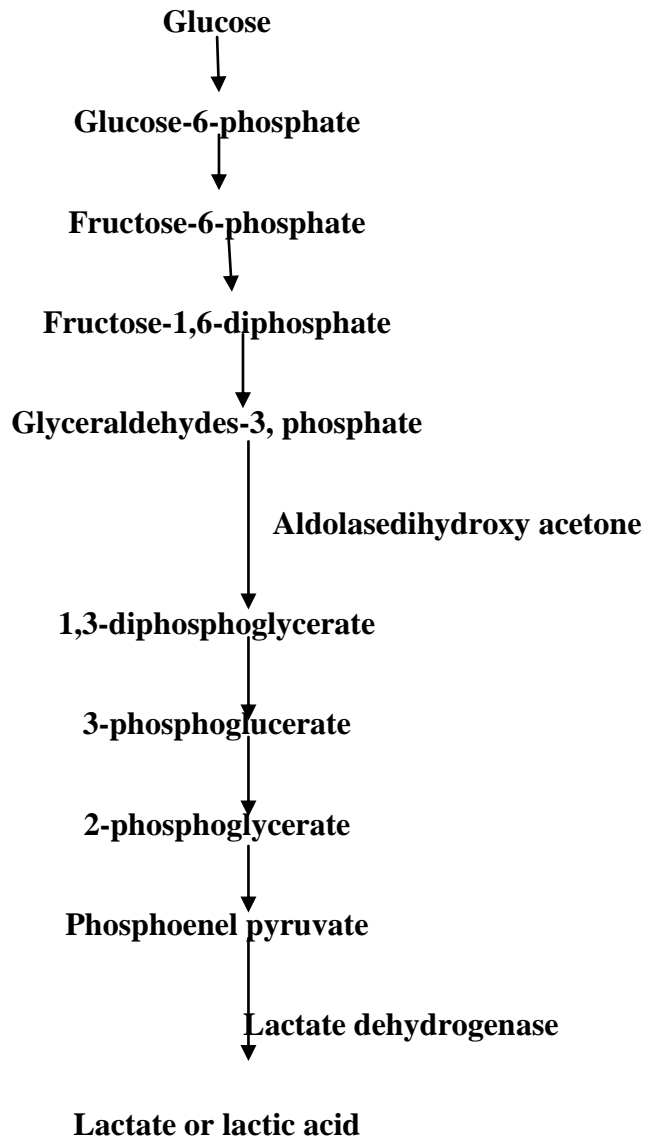
## **2.9 Fermentation**

The production of traditionally fermented milk is widespread throughout Africa. (Mutukumira et al, 1995). Apart from providing nutrients to food, fermented milk also have the advantage of a long shelf life due to its low pH. (Feresu and Nyati, 1990). Fermented milk also have some benefits of the presence of lactase enzyme,  $\beta$ -galactoside for the lactose intolerant consumers, and other inhibitory substances that are effective against several pathogenic spoilage organisms.

Lactic acid fermentation is a metabolic process in which milk sugar, lactose, is oxidised by lactic acid bacteria with the release of energy in the absence of any electron acceptor and in the process lactic acid is produced.(Gadaga et al, 2000) Lactic acid bacteria which are widespread are desirable in food fermentation convert the available sugars to organic acids and lowers the pH of food. Flavour compounds such as diacetyl, acetaldehyde and acetoin are produced which contribute to the desired taste and flavour . Low pH that is produced helps prevent food spoilage and other pathogenic bacteria.

Lactic acid fermentation could be heterofermentation or homofermentation. Homofermentors such as *lactococcus spp*, *Lactobacillus delbrueckii* subsp *bulgaricus*, *Lactobacillus acidophilus*, *Streptococcus thermophilus* convert glucose and they use the enzyme aldolase to produce about ninety percent lactic acid. The lactic acid causes the sharp refreshing test in fermented milk and also preserves milk products. The process occurs as follows:

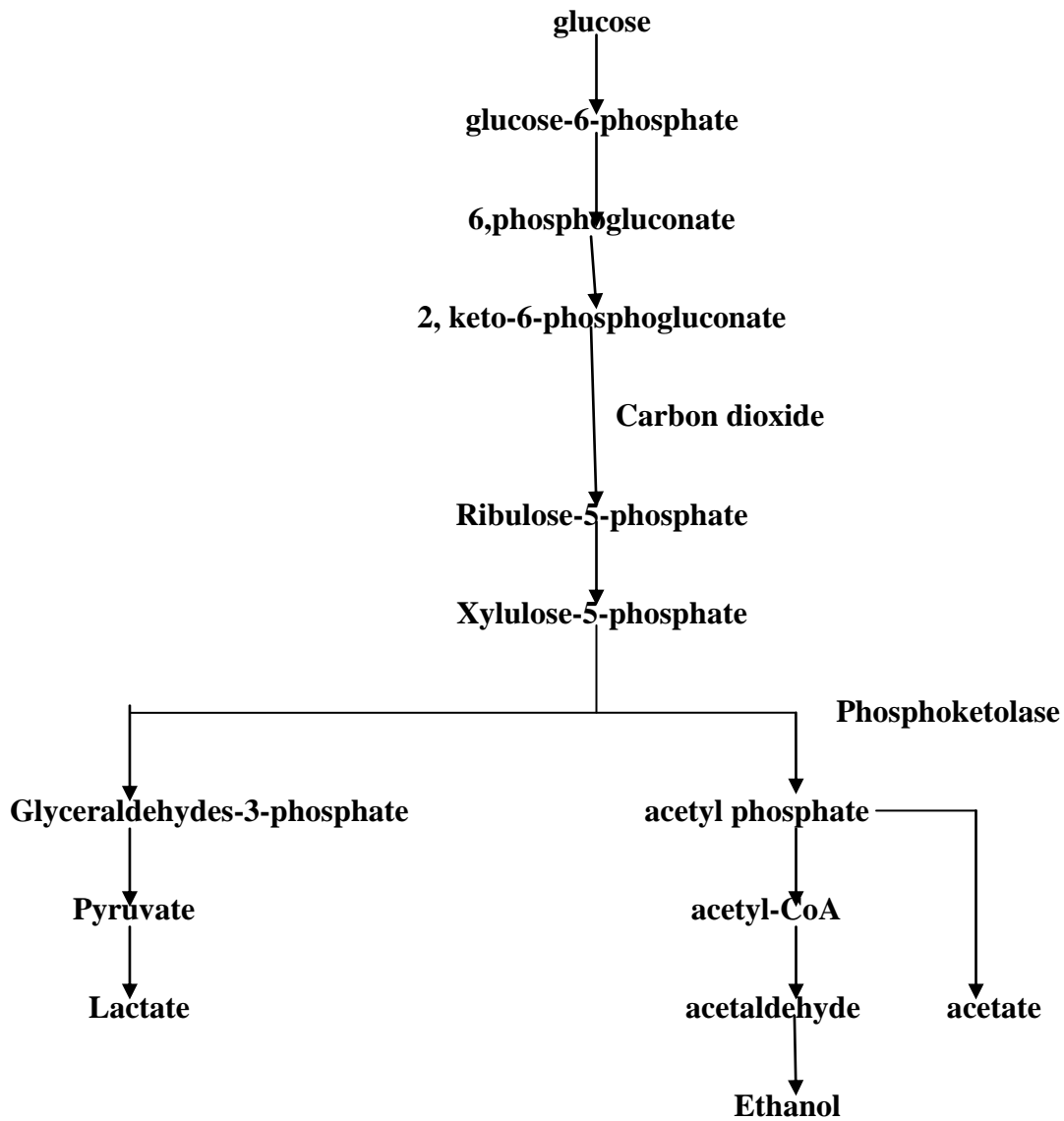




***Homofermentative lactic acid bacteria pathway(Ghandhi, 2006)***

Lactic acid bacteria that cause heterofermentation lack the enzyme aldolase thus cannot use the glycolytic pathway but they use the pentose phosphate pathway. They include *Leuconostoc lactis*. The products of this fermentation are lactic acid, ethanol and carbon dioxide. The overall reaction occurs as follows:





*Heterofermentative lactic acid bacteria pathway (Ganghi, 2006)*

### 2.9.1 FACTORS THAT AFFECT FERMENTATION

Factors could be intrinsic or extrinsic.(Atlas, 1995). Intrinsic factors are those that are characteristic of the food itself or are parameters that are inherent part of the food product ant they include pH, moisture content, water activity, redox potential and nutrient content,(Jay, 2000)

pH of the food is the hydrogen ion concentration in a food. Increasing food acidity by adding a weak base or a weak acid or by fermentation process preserve food. Bacterial growth is

affected by pH of the food and the effect depends on the nature of protein in the food. Charge interaction within amino acids of the polypeptide chain strongly influence the secondary and tertiary structure of the food and folding of the protein(Jay 2000). This affects the active sites of the enzymes, and change in shape of the active site affect the activity of the enzyme. Enzymes are normally inactive at very high or very low ph. Most bacteria grow best at ph ranges around the neutral ph7(from 6.6 to 7.5). lactic acid favours a ph less than 4. This is important with respect to shelf life and spoilage of the fermented foods. (Feresu and Nyati, 1996)

Water is required by bacteria as a solvent for all the biological reactions,(Jay, 2000). Water availability influences bacterial growth. Water can be bound or free water. Bound water is not readily available for use during bacterial growth but free water can be easily used by bacteria for growth and reproduction. Water activity is the ratio of the vapour pressure of a food substance compared to the vapour pressure of pure water at the same temperature,(Jay, 2000).

$a_w = P/P_0$  where  $p$  is the ratio of water vapour pressure of the food substrate and  $P_0$  is the vapour pressure of water at the same temperature. All these are affected by humidity.(Atlas, 1995). Water activity describes the degree to which water is bound in a food thus its availability to participate in a chemical or biological reaction and its ability to participate in microbial growth.

Water activity of less than 0.91 is not favourable for most microorganisms. *Staphylococcus aureus* which causes food poisoning favours a water activity as low as 0.8,(Farber et al, 1992), while *Clostridium botulinum* another food poisoning bacteria do not grow at a water activity less than 0.95(Jay, 1995).

Microorganisms also have varying oxidation-reduction potentials. Redox potential is the ratio of total oxidising (electron accepting) power to total reducing power of a substance,(Morris, 2002). Aerobic microorganisms such as *Bacillus spp* should be oxidised and anaerobic species such as *Clostridium spp* should be reduced. Nutrient requirement vary with microorganisms. The nutrients needed for optimum growth include, water, energy, vitamins and minerals,(Jay 2000). Microorganisms should draw nutrients from the surrounding environments. Carbon is required as the source of energy, nitrogen and sulphur requirements are often met by organic nutrients containing amino acids and proteins as well as peptides. Microorganisms usually require B vitamins in low quantities and almost all food substrate acted upon by microorganisms have an abundant supply of these B vitamins but usually some microorganisms cannot synthesise them for their vitamin supply.

Extrinsic factors are those that are referred to the storage environment such as types of packaging atmosphere, effect of time-temperature conditions and processing steps. (Loss and Hotchkiss, 2002). Temperature is important in the growth of microorganism and all microorganisms have specific temperature ranges for growth with maximum, minimum and optimum temperatures. Four types of microorganisms exist depending on their favourable temperature ranges namely, mesophiles, thermophiles, pscychrophiles and pschrotrophs (Atlas, 1995). Low temperatures reduce the fluidity of membranes and thus restrict transport of essential nutrients and also slow down enzyme reaction rates,(Jay, 2000). Too low temperatures means metabolic reactions cannot occur and at optimum temperatures, growth rate and enzyme reaction rates are at their peak,(Atlas, 1995). High temperatures denature enzymes. Microorganism produces shortest doubling time at their optimum temperatures,(Lund et al 2000, Doyle et al,2000). Mesophiles favour temperature ranges between 30 to 40 °C with an optimum temperature of 30°C and examples of mesophilic lactic acid bacteria include *Lactococcus lactis subspp lactis*. Thermophiles are usually used

in yogurt making and examples of such include *Lactobacillus bulgaricus* and *Streptococcus thermophilus* and their favourable temperatures range from 48 to 50 °C,(Lund et al, 2000)

### **2.9.2 Titratable acidity**

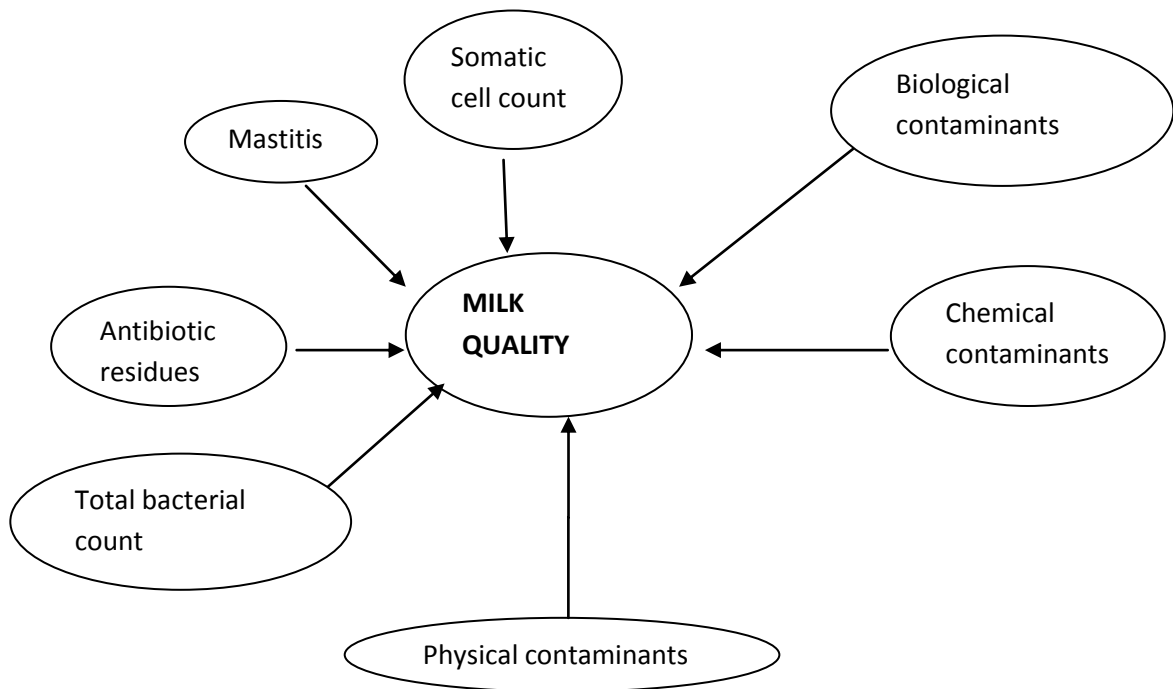
Milk is an amphoteric substances i.e it exhibits both acidic and basic properties. It turns red litmus paper blue and blue litmus paper red. Acidity in milk is caused by two factors which are:(Schmidt et al, 1996)

- Apparent or natural acidity which is due to citrate, phosphates, whey proteins, casein and dissolved carbon dioxide during milking and thereafter.
- Real acidity or developed acidity that occurs from the accumulation of lactic acid that comes from the breakdown of lactose by lactic acid bacteria.

Generally milk acidity means both natural and developed acidity. The acidity of milk is determined by titrating a known volume of milk with a standard alkali and the end point which is determined by an indicator like phenolphthalein. Acidity determined in this way is expressed as percentage lactic acid but the acidity can also be expressed as the concentration of hydrogen ions in milk. The former measures total acidity but does not give the strength of the acid, pH indicate the strength. Fresh milk should have no significant acidity, and acidity of fresh milk ranges from 0.1 to 0.26% and this figure varies with herds. Acidity also changes during lactation probably due to high acidity of colostrums, and also at the end of lactation milk acidity is low (Schmidt et al, 1996). Mild or subclinical mastitis lowers milk acidity. Lactic acid bacteria should increase to several millions before there is a significant rise in Ph. A good starter culture should also increase lactic acid from 0.8 to 1.2 % and should cause a pH drop between 4.3 to 4.5 (Schmidt et al, 1996).

Various factors affect milk acidity and these include the age of milk, bacterial content and protein content (Schmidt et al, 1996). As milk ages, titratable acidity increases and bacterial content increase with age. As protein content of milk increase, titratable acidity also increases. Even at refrigerated temperatures, titratable acidity of milk increase due to undesirable microbial growth (Schmidt, et al, 1996). As bacterial count increase tirtratable acidity also increase and pH decrease thus milk become more unsuitable for producing a milk of desired flavour, odour, appearance and shelf life.

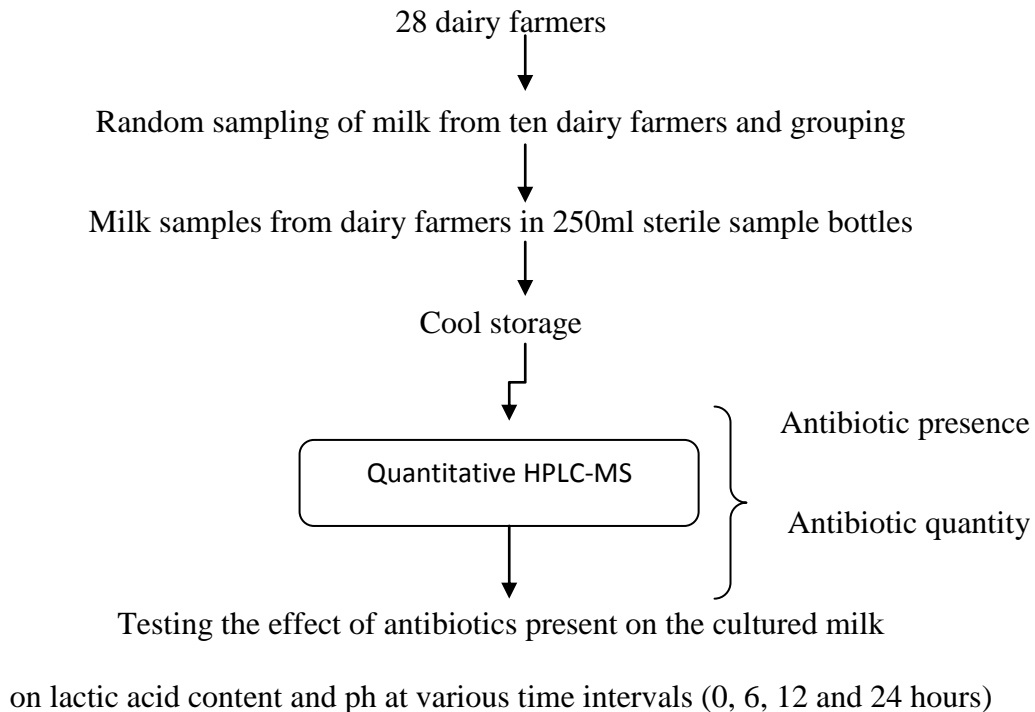
### 2.9.3 CONCEPTUAL FRAMEWORK





## CHAPTER 3: RESEARCH METHODOLOGY

### 3.1 SCHEMATIC DIAGRAM FOR THE RESEARCH METHODOLOGY



### 3.2 RESEARCH DESIGN

Quantitative research design and qualitative design was used. Qualitative research design was done to detect the presence of antibiotic residues. Quantitative research design was used to determine the amounts of and the amounts of antibiotic residues in milk.

Experimental design is used to design experiments for HPLC which was used to design experiments that are going to be done HPLC. Experimental design allows The researcher to test the hypotheses that were stated using an appropriate statistical test.

### 3.3 POPULATION OF STUDY AND THE SAMPLE

The population for the study was raw milk collected at Dairy services. Milk supplied at Dairy Services is supplied in large tankers and this was considered as the population. The milk

samples were then analysed for antibiotic residues. A sample is a subset of the population that should be representative of the overall population. Twenty Samples were collected in 250ml sterile vials for analysis. Samples were analysed twice at Central Veterinary Laboratory in January and February. Ten samples per analysis thus twenty samples were analysed. Ten farmers were selected from those that supplied milk at Dairy services in December.

### 3.4 SAMPLING AND SAMPLE COLLECTION

The samples to be analysed should be collected from all the various groups of dairy farmers which are small scale, medium and large scale. Four for the large scale, three for the medium scale and one for the small scale group. The samples were randomly collected and the samples were separated according to the group the farmer belongs. Ten farmers that supplied milk in December were used. Samples were collected in 250ml sample bottles that were frozen up to the time of analysis. The bottles were sterile and are autoclavable after use to sterilise them. Random sampling was used to avoid bias on the day of sample collection any milk that was present was collected for analysis noting the source which could be small scale, medium or large scale then the samples were analysed for antibiotic residues. The samples were then frozen and a cooler box used to transport the samples to Central Veterinary Laboratory for analysis the same day to avoid changes in milk composition.

FARMER	AVERAGE AMOUNT SUPPLIED(AS AT 31 JANUARY 2014)
A	8 940 LITRES
B	4 345 LITRES
C	191 LITRES
D	642 LITRES
E	820 LITRES
F	646 LITRES

G	890 LITRES
H	2335 LITRES
I	830 LITRES
J	868LITRES

### 3.5 PRINCIPLE OF HPLC

High performance liquid chromatography (HPLC) is used to separate compounds in solution. Gaseous samples can also be separated. Separation is based on partitioning behavior of compounds between the mobile and the stationary phase. The mobile phase must be degassed to eliminate air bubbles. Compounds are separated by passing the mixture through the column. Since compounds have different mobilities they exit the column at different times thus they have different retention times. The HPLC instrument consists of a reservoir for the mobile phase, a pump, injector, a separation column and a detector. The detector is a device that senses the presence of components from the mobile phase and converts it to an electrical signal. For detection of compounds retention times of known compounds are related to unknown compounds and thus detected. The peak and the width of peaks are important. Each peak represents a certain item and standards are used in the identification of the substances. The peak width which is considered under the curve measures the concentration of various substances as explained by Chatwall (2010).

### **3.6 SAMPLE PREPARATION**

#### **3.6.1 Preparation of samples for antibiotics detection using HPLC.**

Agilent 6100 Series Single Quad Liquid Mass Spectrometer system was used. Samples for HPLC should be defatted before analysis. All the chemicals and reagents used were of analytical grade. Only distilled water was used for analysis.

For HPLC- in the extraction of antibiotic residues, milk samples was centrifuged first to precipitate proteins using acetonitrile-methanol, deionized water (40:20:20). Centrifugation is done at 3 000rpm for 10 minutes. The portion remaining on supernatant after proteins were precipitated was used for analysis using HPLC.

HPLC analysis determines the presence of antibiotic residues and their types and their concentrations. The various peaks shows the different types of antibiotics present which were then compared with standards for various possible antibiotics which can be used in treating bacterial infections in milking cows. The procedure used was adapted from the Lab manual for analysis of food and antibiotic residues of India (2012) for antibiotic residues.

#### **3.7 Testing the effect of antibiotics present on milk fermentation**

Milk was pasteurised at sixty-five °C for thirty minutes. After cooling to room temperatures various concentrations of the albendazole antibiotic were added to five-hundred ml of the pasteurised milk. Forty mls of lacto were used as the culture in each mixture of antibiotic and pasteurized milk as follows.

Albendazole concentration used( $\mu$ L)	Raw milk used(ml)	Lacto used(ml)
0	500	40
100	500	40
500	500	40
750	500	40
1000	500	40
2000	500	40

Back fermentation was used to culture raw milk at thirty-five °C. Titratable acidity and pH were measured at 0, 6, 12 and 24 hour intervals. pH was measured using a pH meter (version HI 98128) from HANNA Instruments and titratable acidity was determined using phenolphthalein indicator and freshly prepared 0.1 N sodium hydroxide.

### **3.8 Validity and Reliability**

All equipment used was calibrated. All reagents used were of analytical grade. Sampling was random to avoid bias on the results. Sample analysis was done in duplicate to avoid bias. Averages of the samples were then used. Also sampling was done three times to make sure that results obtained were accurate and to avoid bias and seasonal variation. Thus results and conclusions drawn were valid and reliable.

### **3.9 Data presentation and Analysis**

Graphs to compare the levels of antibiotic residues in milk from various groups of farmers. Graphs were also used to compare lactic acid content, pH, and antibiotic concentration.

Software packages were used. T test statistics and ANOVA were used to compare the various means for antibiotic residues in various milk samples. One way ANOVAs was used.

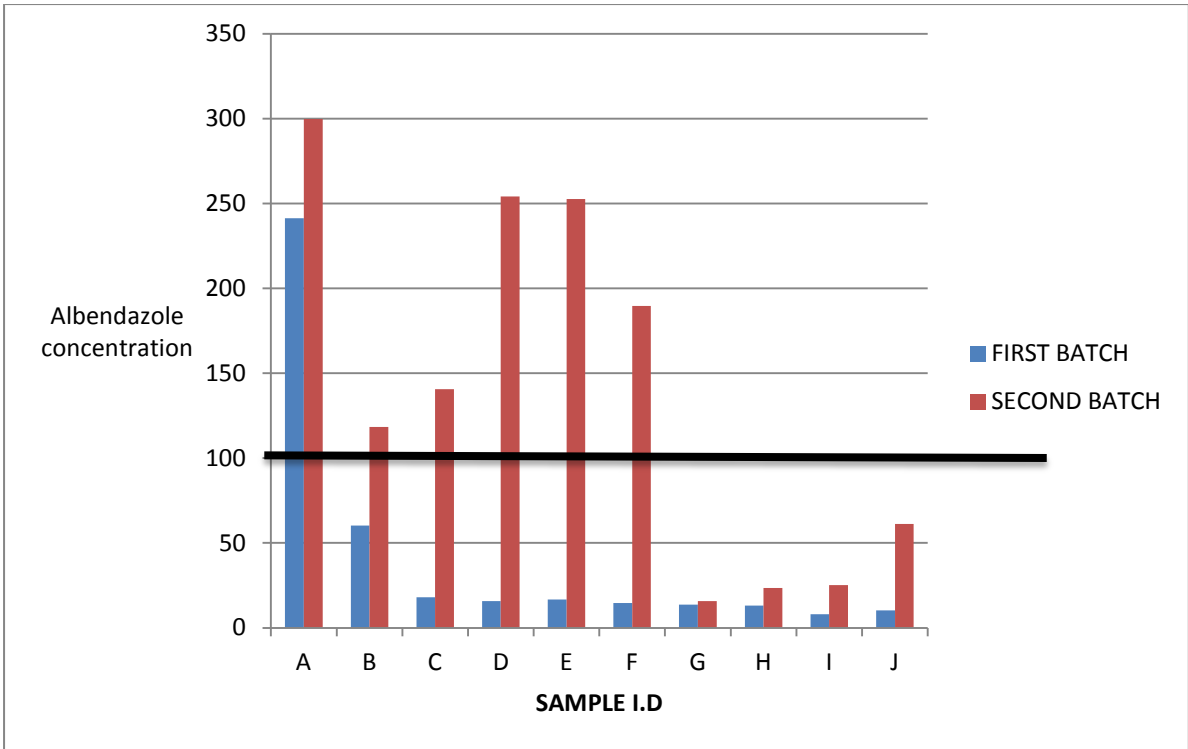
## Chapter 4 Results

**Table 4.1 –Qualitative HPLC Analysis**

Sam ple ID	Albendazole	Thiabendazole	Amoxicillin	Penicillin G	Enrofloxacin	Dexamethasone	Sulfachloropyridazine	Sulfadiazine	Sulfamethazine	Sulfamethizole	Sulfamethoxazole	Sulfamethoxine	Sulfaquinoxaline	Trimethoprim
A	X	O	O	O	O	O	O	O	O	O	O	O	O	O
B	X	O	O	O	O	O	O	O	O	O	O	O	O	O
C	X	O	O	O	O	O	O	O	O	O	O	O	O	O
D	X	O	O	O	O	O	O	O	O	O	O	O	O	O
E	X	O	O	O	O	O	O	O	O	O	O	O	O	O
F	X	O	O	O	O	O	O	O	O	O	O	O	O	O
G	X	O	O	O	O	O	O	O	O	O	O	O	O	O
H	X	O	O	O	O	O	O	O	O	O	O	O	O	O
I	X	O	O	O	O	O	O	O	O	O	O	O	O	O
J	X	O	O	O	O	O	O	O	O	O	O	O	O	O

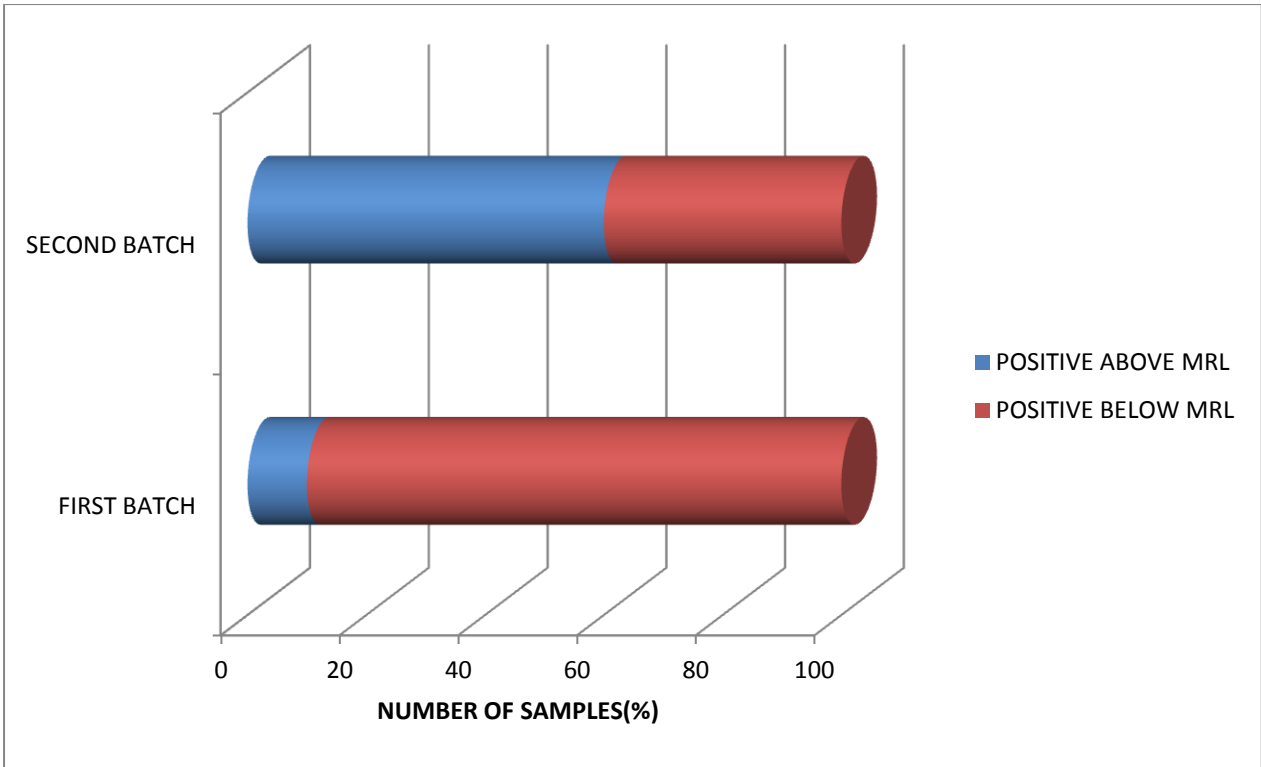
**Qualitative Analysis Results (Screening):**      **Key: X = Positive    O = Not Detected**

Results of Qualitative HPLC-MS shows that from the fourteen antibiotics that were analyzed only one antibiotic was present which is albendazole. All the other antibiotics were not detectable on the HPLC-MS machine.



**Fig 4.1 Albendazole concentrations obtained on the HPLC machine**

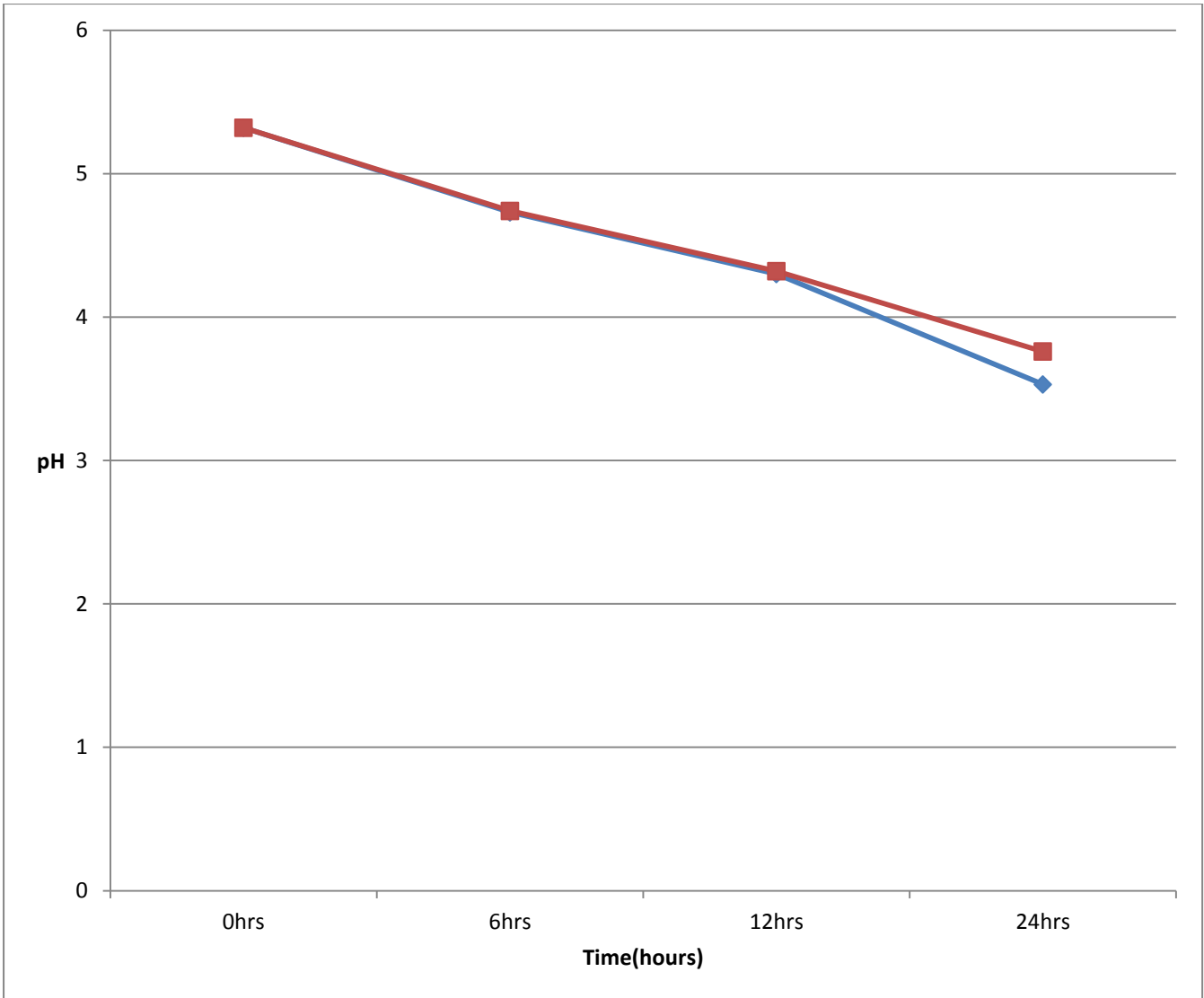
Fig 4.1 shows the antibiotic albendazole levels in the ten samples that were analyzed. Fig 4.1 shows that during the first analysis farmer A had concentration of antibiotic albendazole above the Codex Alimentarius standard which is 100ppb. Nine samples were below the codex standards. On the second batch, the graph shows that six samples were above the Codex Alimentarius standards and only four samples were below the Codex standards. The samples which were above MRLs on the second analysis A, B, C, D, E and F with farmer A having the highest antibiotic concentration.



**Fig 4. 2: Summary for albendazole concentrations**

The analysis was done to detect the quantities and to see if the antibiotics present were within acceptable limits using the Codex standards. The results showed that all the samples had the drug but at different concentrations. During the first analysis those that had antibiotics above constituted only ten percent and ninety percent had antibiotic residues below the maximum residual limit (MRL). However on the second analysis sixty percent of the antibiotics were above maximum residual limits. Above all the antibiotics should not be present in milk.



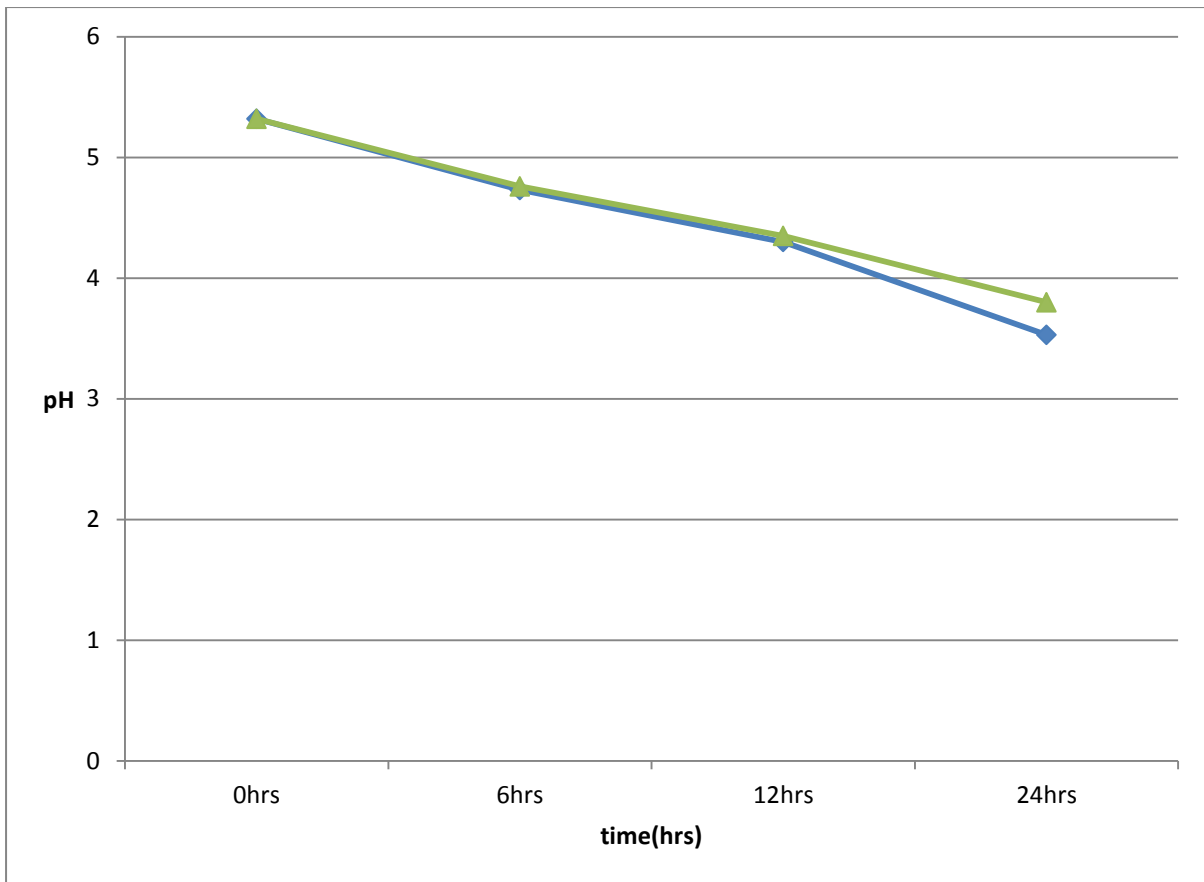


**KEY:** Blue line-control

Red line 100µl albendazole

**Fig 4.3: Comparison of no albendazole added with 100µL albendazole.**

Both samples started at pH 5.32. After twenty-four hours the sample that had no antibiotic had decreased in pH to 3.53. The sample that had 100µL added had decreased its pH up to 3.76 as indicated fig 4.3.

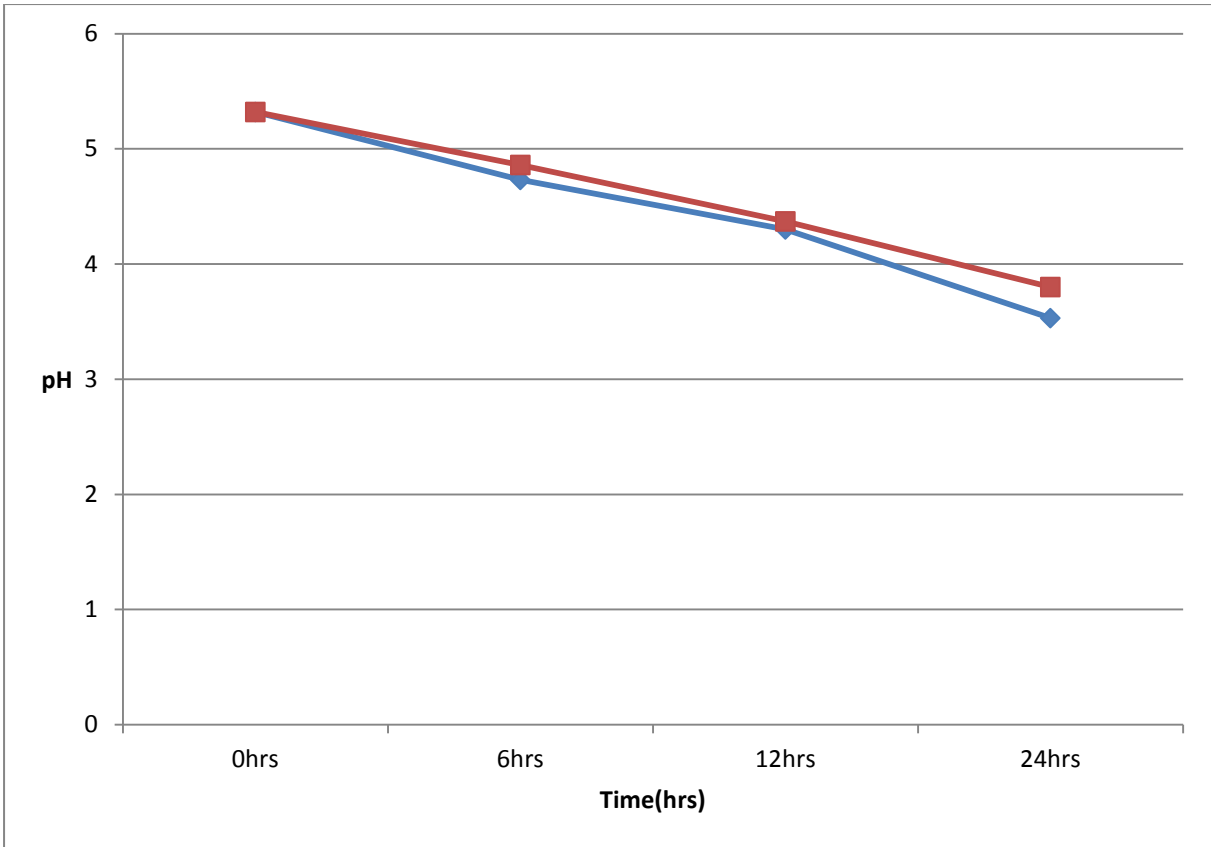


**KEY:** Blue line- no albendazole

Green line-500μL albendazole

**Fig 4.4: Comparison of pH change of no albendazole with 500 μL albendazole**

From fig 4.4 the pH for the sample that had no antibiotic again experienced more pH change than the sample that had 500μL of albendazole. The sample that had no albendazole changed its pH from 5.32 to 3.53. The sample with 500μl albendazole changed at a slower rate such that by twenty four hours the pH was now 3.78.

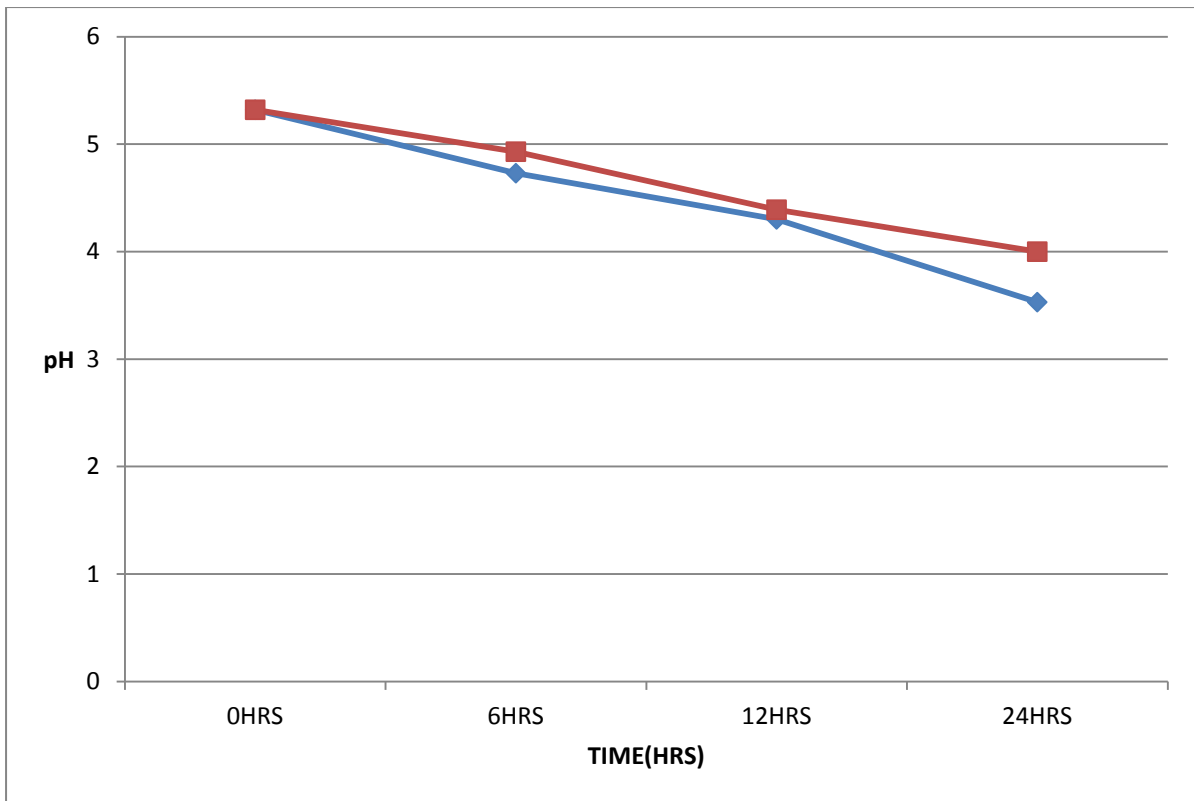


**KEY:** Blue line- no albendazole added

Red line- 750µL albendazole

**Fig 4.5: Comparison of pH change at 750µL albendazole added compared with no albendazole added.**

Both sample experienced a change in pH but the sample with 750µl changed at a slower rate that its pH changed from 5.32 to 3.86. The sample that had no antibiotic changed pH from 5.32 to 3.53.

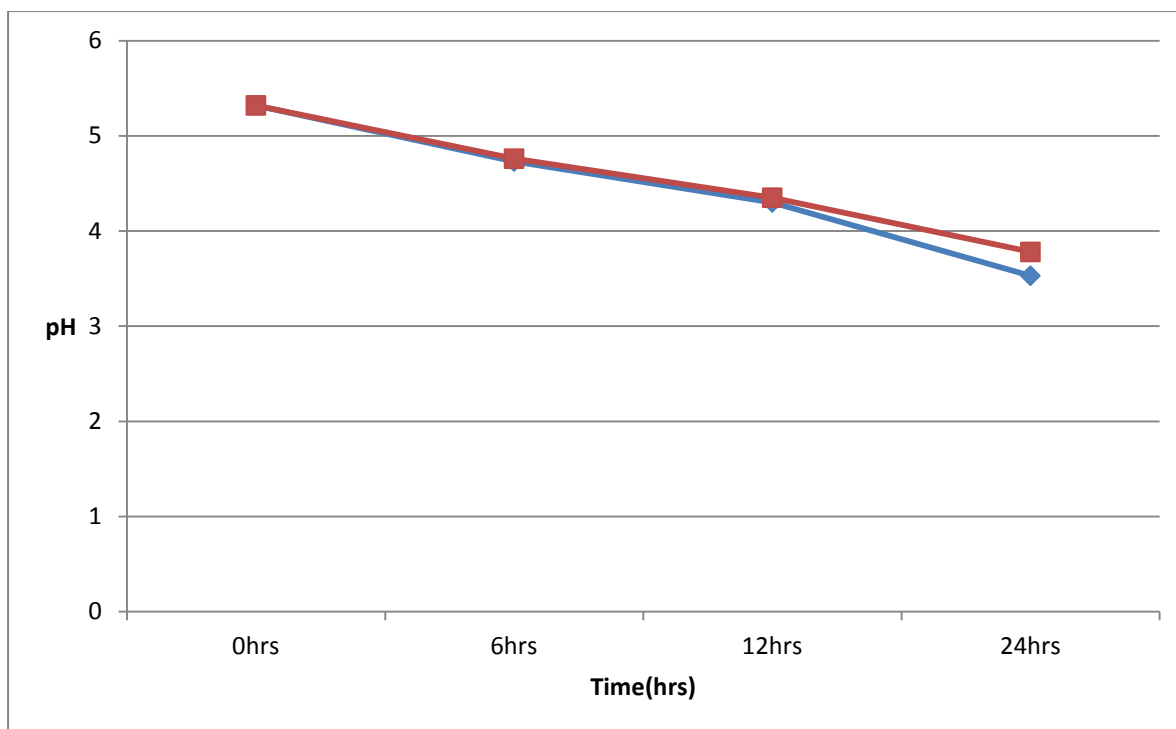


**KEY:** Blue line- no albendazole added

Red line-1000µL albendazole

**Fig 4.6: Comparison of pH change at no albendazole and 1000µL albendazole added**

Fig 4.6 shows that the pH for the samples decreased but the sample that had no albendazole added decreased at a faster rate than the sample that had albendazole added. After the addition of 1000µL albendazole pH changed from 5.32 to 4. In the sample that had no albendazole pH changed from 5.32 to 3.53.



**KEY:** blue line- no albendazole added

Red line- 2000µL albendazole added

**Fig 4.7 Comparison of pH change between no albendazole added and at 2000µL albendazole**

The graph shows that after the addition of 2000µL pH changed from 5.32 to 4.24. Compared with all the samples, this recorded the least change in pH. The graph for the sample where no albendazole was added showed a pH change from 5.32 to 3.53.

#### 4.8 HYPOTHESIS TESTING

**H<sub>0</sub>1: there is no significant difference in antibiotic residues among farmers that supply milk to Dairy processing companies.**

Using Graph pad prism:

A one sample t-test was used to test if the values obtained differ significantly from the maximum residual limit of 100µg/litre.

$$t_{\text{calculated}} = 0.4164$$

from the tables at 5% significant level with 9degrees of freedom (n-1)

$$t_{\text{tabulated}} = 2.262$$

Decision:  $t_{\text{calculated}} (0.4164) < t_{\text{tabulated}}(2.262)$ , therefore we accept  $H_0$  and conclude that there is no significant variation in antibiotic concentration among farmers who supply milk to Dairy services.

**H<sub>2</sub>: There will be no significant differences in lactic acid content in milk treated with various antibiotic concentrations.**

**Table 4.2: summary of results one way ANOVA**

Table Analyzed				
Data 2				
One-way analysis of variance				
P value	P<0.0001			
P value summary	***			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	4			
F	153.9			
R squared	0.9585			
Bartlett's test for equal variances				
Bartlett's statistic (corrected)				
P value				
P value summary	ns			
Do the variances differ signif. (P < 0.05)	No			

ANOVA Table	SS	Df	MS	
Treatment (between columns)	0.8565	3	0.2855	
Residual (within columns)	0.03711	20	0.001856	
Total	0.8936	23		

$$F_{\text{calculated}} = 153.9$$

$$F_{\text{critical}} (3:20)$$

At 1% significance level=4.94

At 5% significance level=3.10

Decision if  $F_{\text{calculated}} > F_{\text{tabulated}}$  we reject  $H_0$  and conclude that the percentage lactic acid vary significantly with different antibiotic concentrations.

**Table 4.3: To see if there is a correlation between albendazole concentration and percentage lactic acid**

Number of XY Pairs	6	6
Pearson r	0.9842	0.9522
95% confidence interval	0.8578 to 0.9983	0.6186 to 0.9949
P value (two-tailed)	0.0004	0.0034
P value summary	***	**
Is the correlation significant? (alpha=0.05)	Yes	Yes
R squared	0.9687	0.9067

From the correlation analysis using Graph pad prism, there is a strong positive correlation from the  $R^2$  value obtained (0.9687).

## 4.9 DISCUSSION

Total milk produced by farmers by end of January was 20 507litres. Farmer A on average produced 8940 liters which constituted 43.4% of the total milk produced by the farmers during the sampling period. This meant Farmer A had the highest impact on the quality of milk as he had the highest level of antibiotic on both analysis. On the second analysis, total milk produced by farmers (A, B, C, D, E and F) was 15 584litres and this constituted 76% of the total milk produced. On the second analysis farmers (A, B, C, D, E, and F) had albendazole concentrations above the Codex Maximum Residual Limit of 100ppb.

From the HPLC-MS results only one antibiotic was detectable. Mass spectroscopy detector is a powerful tool which can be coupled to HPLC to give high detection limits. The method provides a higher sensitivity and selectivity enabling less extensive sample preparation, greater sensitivity via increased selectivity, (Fallah, 2010). It allows the use of shorter HPLC columns and this enables shorter run times and higher sample through put. These translate time and effort saving as well as providing a more sensitive method. The HPLC Agilent 6100 Series has a detection limit of 4ppb that has been set for all the antibiotics that were analyzed, the detection limits for all the antibiotics that were analyzed varied between 2-5 ppb thus a detection limit of 4ppb has been set to cater for variations for all the antibiotics that were analyzed.

From the hypothesis testing, there is no significant difference in antibiotic concentrations among Dairy farmers. All farmers tested positive for albendazole and this means that even though there was variation in the levels of antibiotics, all farmers tested positive for the antibiotic albendazole. Also there was a significant difference in the levels of lactic acid among fermented milk treated with different antibiotic concentrations. Even though albendazole did not stop the fermentation of milk, there is however a significant difference in the level of lactic acid and pH of fermented milk treated with different antibiotics. Also there



was a strong correlation ( $R^2$  of 0.97) between the percentage lactic acid and antibiotic concentration. As antibiotic concentration increases, the rate of fermentation decreased as shown by a decrease in pH with time.

Zubeir and Owni (2009) did a study in Khatoum state to detect antibiotics in milk. Their findings were that farmers used to mix milk in bulk tankers thus diluting the amounts of antibiotics present in the milk. In this research, sulphonamides were detected in these milk samples. In this research also, antibiotics were used to prolong the shelf life of and to preserve milk. Antibiotics were also present in milk because the studies were done in winter were the cold weather causes development of diseases such as pneumonia and farmers used antibiotics to treat cattle conditions and these were transferred in milk.

Zhao et al, (2005) also did a research to determine antibiotic residues in milk. Findings were that 14.3% of samples were contaminated with ox tetracycline residues above WHO standards. In this research, thirty percent of samples sterilized had tetracycline residues. This shows that antibiotics are resistant to heat thus they are present in ultra heat treated milk. The presence of antibiotics could have been attributed by the use of tetracyclines as feed additive and also to prevent diseases such as mastitis.

36.5% of sample analyzed had  $\beta$ -Lactam residues in studies done in Pakistan by Khaskeli et al, 2008. In Turkey, Ankara region 5.5% of samples was positive for antibiotic residues in studies done by Ceyhan and Bozkurt, (1987). This shows that there is a possibility of development of antibiotic resistant strains since these antibiotics have been since used for a long time. Deme et al, (1992) did a research on the detection of antibiotic residues in milk samples and out of the fifty milk samples analyzed six were positive for pencillin G. Aydin et al, (1989) discovered that forty four percent of the samples analyzed were contaminated with antibiotics. Wab, (1997) did a research on antibiotic residues in Trinidad and 10.8% were

positive in both unprocessed and processed milk. In Kenya also, Sasany et al, (2008) did a research on antibiotic residues in milk. Findings were that from three hundred and eight four cows sampled, thirteen percent were positive for penicillin G. according to a research carried out by Shitandi (2001) in Kenya also, twenty one percent of the 1109 cows analyzed were positive. This shows a reduction in the use of antibiotics and probably an indication of the increase in awareness of farmers on the harmful effects of antibiotics in both the quality of milk and to human health.

In Kuwait, 5.4% of imported pasteurized milk were positive and above 4ppb permissible Codex Maximum residual limit. In all the studies the amount of antibiotic residue in milk usually differ depending on the level of effectiveness of legislation and the method used as each method has a specific detection limit. The method of adoption and of implementing the legislation also differs with various states and this affect the amount of residues present.

Very few studies have dealt with the antibiotic albendazole because it does not inhibit fermentation but it just slows down the rate of fermentation. The maximum permissible amount of albendazole according to the Codex Alimentarius Commission is 100ppb. Maximum residual limit is the maximum concentration resulting from the use of veterinary drugs that is recommended by the Codex Alimentarius Commission to be legally permitted or recognized as acceptable in or on a food item that is based on the amount and type of residues considered without toxicological hazard for human health as expressed by the Acceptable Daily Intake (ADI), or on the basis of a temporary acceptable daily intake that utilizes an additional safety factor (WHO/FAO, 1989). It also takes into account the relevant public health risks as well as food technological aspects and estimated food intakes. When establishing MRLs consideration is also given to residues that occur in food of plant origin or and in the environment. MRLs may be reduced to be consistent with good practices in the use

of veterinary drugs and the extent that practical analytical methods are available, (Turnspeed et al, 2010).

For albendazole according to Joint FAO and WHO conference of 1989 the factors which were considered in setting up the maximum residual limits for albendazole were as follows:

- The concentration of the total residues is about fifty percent greater in the liver than either muscle or fat
- Estimated maximum daily intake of residue at four days withdrawal time or longer does not exceed ADI.
- Concentration of total residues at ten days withdrawal time or longer does not exceed 5mg/kg in the liver or kidney or 0.1mg/kg in the muscle or fat
- The residues at six days withdrawal time or longer contain low concentration of toxic substances. there are no residues of parent drug and only small concentrations of the sulphoxide
- More than ninety five percent of the residues at four days withdrawal time or longer are bound residues of which less than fifteen percent are bioavailable.
- A withdrawal time of ten days is long enough to for the drug to be efficacious and is compatible with good animal husbandry practices.
- Concentration of residues in milk declines rapidly following drug administration in less than 0.1mg/ litre by the fourth milking.
- Amounts of residues in milk declines rapidly following drug administration and less than 0.1 mg/L by the fourth milking.

Albendazole is a helminthic antibiotic drug used for treating flatworms (flukes and trematodes), fasciolosis, tapeworms and nematodes. It is a dewormer and its oral suspension

can be used in sheep, goats and cattle to control liver fluke, tapeworms, stomach worms, intestinal worms and lung worms (Maing et al, 2001).

If the drug has been administered to cattle, they should not be slaughtered within twenty-seven days of slaughter. Sheep and goats must not be slaughtered within seven days of treatment. Albendazole should not be used in breeding age female dairy cattle and also it should not be used in lactating cows because withdrawal periods in milk have not been established. Maing et al, (2010) explained that it should be sixty hours and Turnspeed et al (2010) indicated that it should be one hundred and twenty hours. On a label downloaded on the internet from the manufacturer, Ravensdown, the withdrawal time for albendazole is ten days. Ravensdown indicated that it is an offence to sell milk or meat for human consumption from an animal just treated with the drug albendazole. It should not be administered to female cattle for the first forty five days of pregnancy or for forty five days after removal of bulls.

Experience with albendazole in humans extends to more than twenty years and it is remarkable how adverse effects have been reported, (Gil- Grande, 1993). Albendazole does not usually have serious life threatening effects recorded in humans at doses used to treat intestinal helminths. It appears that most problems that occur with albendazole occur at higher doses treatment for systematic infections where problems of drug-parasite interaction occur and causes high specific syndromes such as liver echinococcosis, or central nervous symptoms in neurocysticercosis (Gil-Grande et al, 1993). Fatalities have almost entirely associated with long term, high dose treatment in AIDS related infections and have been due to underlying disease rather than the effects of the drug itself.

Albendazole causes bone marrow suppression, aplastic anaemia, and agranulocytosis in humans without underlying hepatic dysfunction. Patients with liver diseases, including hepatic echinococcosis, appear to be at more risk for bone marrow suppression. Albendazole

also causes a decrease in blood cell counts thus should not be administered to patient with a low blood cell count. Also exposure to albendazole in pregnant women is crucial as it has adverse effects to the fetus. Women taking the drug as soon as they become pregnant should stop using the drug immediately. If pregnant while taking the drug, the women should be apprised of the potential hazard to the fetus.

Various studies were done to assess the various effects of the drug albendazole and the studies were done on animals, humans and rats and rabbits and the various findings are indicated bellow. A long established anthelmthic drug for the bore human and veterinary use having a broad spectrum of indications.

Albendazole has minor to moderate adverse effects(headache, fever, gastrointestinal upset, body aches, severe scrotal aches and shock).Albendazole is erratically absorbed from the intestine, being dependent on the type of meal and pH of the stomach, is then metabolized to its active ABSX, which is metabolized to non-active Albendazole sulphone. Pharmacological data.-20-30% of ingested albendazole in rats and mice, 1% in humans, and 50% in cattle was absorbed. Oral dosing produced very low plasma levels of unchanged drug because of rapid first pass metabolism in the liver (WHO/FAO, 1989). Metabolic reactions are the oxidation of the sulphide moiety of ABN to the sulfoxide and sulphone moiety, followed cleavage of the carbamate moiety to form the 2-aminosulfone which to the main residue in the livers of sheep and cattle, rats, mice, humans.

In studies which curb were administered to mice for twenty five months, symptoms noted included, anaemia, leucopenia, and testicular degradation at 400mg/kg of body weight/day(WHO/FAO,1989) and also hepatocellular vacuolation at 100 and 400mg/kg of body weight/day occurred in rats. In rats for twenty-eight months at 20mg/kg per day caused mortality neutropenia, hyper chlostrolamia, testicular degeneration and hepatic fatty metamorphosis (WHO/FAO, 1989).

Also noted were incidence of endometrial/cervical tumours and histolytic sarcoma on the skin. Also albendazole do not produce bacterial mutations, chromosomal or morphological transformation in cultured mammalian cells. In rats no effect on fertility but reduction in postnatal survival and growth of fetus, in pregnant dams there is decrease in survival, low birth weight and depressed growth during suckling of pups 400 mg/day/kg body weight. Albendazole produced negative results in bacterial mutation tests using strains TA1530, TA1532, TA1534, TA1537, TA98, TA100, LT2 his- and G46 of *Salmonella typhimurium*, (WHO/FAO, 1989). It produced no clastogenicity in an in vitro metaphase analysis of Chinese hamster ovary (CHO) cells, and was negative in an in vitro cell transformation assay in BALB/3T3 mouse cells (WHO/FAO, 1989). However, an in vivo mouse bone marrow micronucleus test on albendazole, which had been isolated from a formulated product, gave a positive result. This result indicated that albendazole was an in vivo somatic cell mutagen. In the absence of any tests on germ cells, it remains unclear whether or not albendazole can induce heritable mutations. The results of the mutagenicity tests on albendazole and those on netobimin and albendazole sulphoxide were consistent with these substances all being in vivo aneugens (WHO/FAO, 1989).

The various concentrations of albendazole obtained in the milk as antibiotic residues depend on the amount administered to dairy cattle by different farmers. The concentrations also occur depending on time lapse after the administration as the concentration in milk changes with time after the administration of the antibiotic. Due to the need for money farmers are usually not following the withdrawal times for the antibiotic residues. At times farmers may not keep a record so that there is a correct estimation of time after the administration of the antibiotic thus should follow proper withdrawal times by proper record keeping. At times is also necessary to withhold milk from treated quarters alone from that treated with the antibiotic.

The worms that usually affect animals their rate is high during the periods when animals feed with grass and other feeds rather than when they are fed during the time when they are given purchased feeds,(Maing et al,1998).

The rate of lactic acid production increase with time. This is because the amounts of microorganisms present in the starter culture replicate by binary fusion. As more microorganisms are produced, more lactic acid is produced from the fermentation process. pH decrease with an increase in the production of lactic acid. The presence of albendazole slows down the activity of the starter culture. Albendazole disturbs the whole process of energy production by limiting glucose uptake by cells (Maing et al, 1998). This means that the growth of microorganisms and multiplication is affected and thus production of lactic acid is affected. As the antibiotic content increase rate of production of lactic increase and thus there is a slight decrease in pH. The change in lactic acid content and pH are all affected by the presence of the antibiotic residues in the sample. Acidity in milk is caused by two factors which are :( Schmidt et al, 1996)

- Apparent or natural acidity which is due to citrate, phosphates, whey proteins, casein and dissolved carbon dioxide during milking and thereafter.
- Real acidity or developed acidity that occurs from the accumulation of lactic acid that comes from the breakdown of lactose by lactic acid bacteria.

Generally acidity means both developed and total acidity present. pH also measures the strength of the acidity. As antibiotic concentration increase , the strength of the acid decreases in fermented milk.

In this chapter data has been analysed and hypothesis tested and also discussion of results.

## **CHAPTER 5: SUMMARY, CONCLUSION AND RECOMMENDATIONS**

### **5.1 SUMMARY**

Chapter one of the research explained that food safety was important and covers various issues. In chapter one it was indicated that there is need to prevent physical, chemical and biological contaminants in food. Chapter one also explained that Dairy companies only test for major groups of antibiotics in foods and also quantitation methods are not used. It was also highlighted that the presence of antibiotic residues pose a serious health threat to the consumer as exposure to antibiotic residues may eventually cause antibiotic resistant in individuals. Antibiotics residues in milk also affect the activities of starter cultures in Dairy fermented product such as cheese, yorghut and also fermented milk.

In chapter two, various scholarly articles relating to the occurrence of antibiotic residues in milk were outlined. Different authors who investigated, explained, highlighted or observed anything to do with the occurrence of antibiotic residues in milk were indicated in chapter two. Various methods for detecting antibiotics as well as the various groups of antibiotics and their maximum residual limit in various foods according to the Codex Alimentarius Commission were indicated.

The research methodology was outlined in chapter three. Experimental procedures were also highlighted here. Details of the population, how samples were selected, sampling plan, were indicated. Also explained was sample preservation up to the time of analysis and also preparation for the HPLC method. Details of statistical analysis were explained also. In chapter three also, it was explained how the effect of various antibiotic concentration on cultures was to be determined.



Data presentation and analysis was done in chapter four. Graphs were used to present data and tables were also used. Statistical package , Graphpad prism was used to do statistical analysis. T –test and one way ANOVA were used to analyze data hypothetically.

## **5.2 Conclusion**

All the farmers use the antibiotic albendazole often. Farmers do not use the major antibiotics which are known on their lactating cows or they adhere properly to the withdrawal periods as these were not detectable on the HPLC analysis. The levels of the antibiotic albendazole which is an antihelminthic agent in treating worms were high among all the groups of farmers.

The antibiotic albendazole has the effect of slowing down the growth and the activities of starter cultures. It causes a slow decrease in pH than the fermentation of milk with no antibiotic in it. As the antibiotic concentration in milk increase, there is a decrease in the percentage lactic acid produced in milk.

## **5.3 Recommendations**

- There is need for the detection and quantitation of various groups of antibiotics in Dairy industry.
- Thus Dairy companies should procure better equipment which improves the detection and quantitation of milk.
- There is also a need to educate farmers on the groups of antibiotics and their effects on the quality of milk as well as the effect of antibiotic residues on humans.
- There is also need to test oncoming milk before mixing the milk with other milk tankers received.

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**APPENDIX 1: RESULTS OF HPLC-MS**

SAMPLE CODE	FIRST ANALYSIS	SECOND ANALYSIS	CODEX MRL
A	241.31 ±2.22	299.8±2.22	100ng/ml(ppb)
B	60.19±2.22	118.34±2.22	100ng/ml(ppb)
C	17.98±2.22	140.63±2.22	100ng/ml(ppb)
D	15.67±2.22	254.13±2.22	100ng/ml(ppb)
E	16.67±2.22	252.57±2.22	100ng/ml(ppb)
F	14.71±2.22	189.62±2.22	100ng/ml(ppb)
G	13.67±2.22	15.69±2.22	100ng/ml(ppb)
H	13.05±2.22	23.46±2.22	100ng/ml(ppb)
I	8.02±2.22	25.21±2.22	100ng/ml(ppb)
J	10.32±2.22	61.18±2.22	100ng/ml(ppb)

**APPENDIX 2: RESULTS FOR TITRATABLE ACIDITY AND pH OBTAINED WITH VARIOUS ANTIBIOTIC CONCENTRATIONS**

Antibiotic [μL]	0HRS		6HOURS		12HOURS		24HOURS	
	pH	%L.A	pH	%L.A	pH	%L.A	pH	%L.A
0μL	5.32	0.100	4.73	0.315	4.30	0.513	3.93	0.657
100μL	5.32	0.100	4.74	0.270	4.32	0.459	3.76	0.630
500μL	5.32	0.100	4.76	0.252	4.35	0.432	3.78	0.621
750μL	5.32	0.100	4.86	0.234	4.37	0.405	3.80	0.612
1000μL	5.32	0.100	4.93	0.225	4.39	0.360	4.00	0.576
2000μL	5.32	0.100	5.06	0.198	4.43	0.342	4.24	0.540

% Titratable Acidity =

$$\frac{Ml \times N \times 90 \times 100}{V \times 1000}$$

WHERE

Ml = ml of 0.1 N sodium hydroxide used

N = Normality of 0.1 N sodium hydroxide

V = ml of milk solution used

Titratable acidity is the percentage lactic acid in milk

**APPENDIX 3: SUMMARY OF RESULTS FOR SAMPLES WHOSE SAMPLES WERE DETACTABLE BY ANTIBIOTICS**

ANALYSIS	POSITIVE ABOVE MRL	POSITIVE BELOW MRL	TOTAL
FIRST	1	9	10
SECOND	6	4	10

**APPENDIX 4: CALCULATION OF MASS OF SODIUM HYDROXIDE USED TO MAKE 0.1 N SODIUM HYDROXIDE**

Mr of sodium hydroxide

AR of sodium + Ar of oxygen + Ar of Hydrogen

$$=23+16+1$$

$$=40$$

Moles = mass / Mr

$$0.1 \text{ N} = x/40$$

$$0.1 \times 40 = x$$

Thus 4g of sodium hydroxide granules are added to 1000ml distilled water

Appendix 5: Albendazole calibration curve

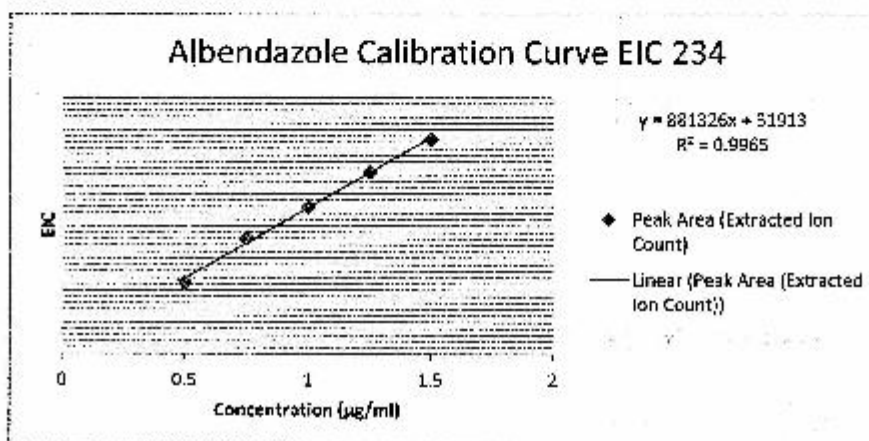
Peak Areas of Albendazole Calibrators at 5 Levels

EIC 234

Concentration in µg/ml Calibrators (standards)

Peak Area (Extracted Ion Count)

0.5	447203
0.75	722618
1	917235
1.25	1137940
1.5	1341200



Results of the samples

First Batch

Sample ID	Peak Area	Concentration	Conce. in Actual Samples ng/ml
A	2158670	2.413133165	241.31
B	562419	0.601940712	60.19
C	190368	0.179791587	17.98
D	169461	0.156069377	15.61
E	134801	0.116742272	11.67
F	161578	0.1471249	14.71
G	152378	0.136686084	13.67
H	146922	0.130495413	13.05
I	102556	0.080155357	8.02
J	122882	0.103218332	10.32



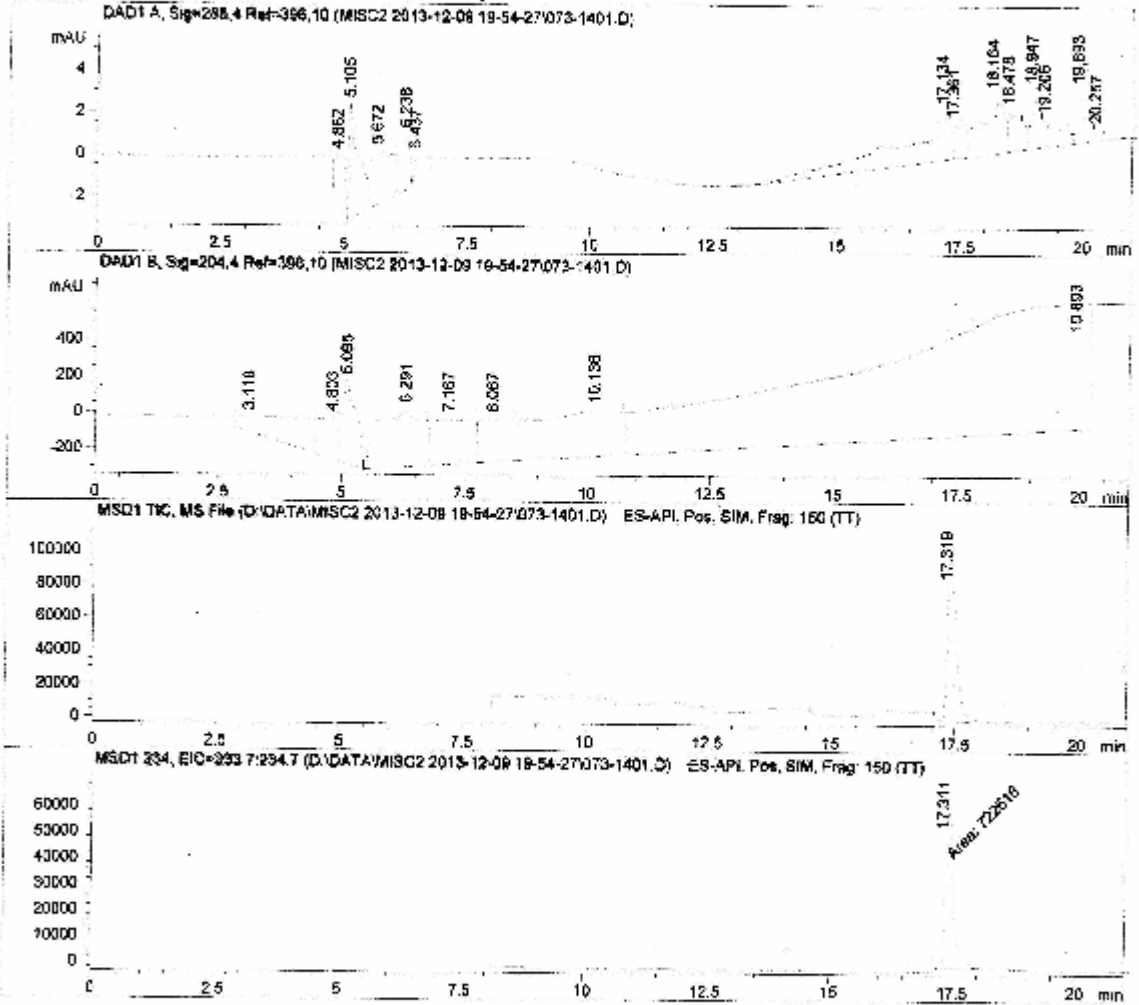


Data file D:\DATA\MISC2 2013-12-09 19-54-27\073-1401.D  
 Sample Name: Solv Cal MS 3

*ALBENDAZOLE CALIBRATOR 2 (STANDARD - conc 0.75 µg/ml)*

Acq. Operator : Tungmiray Mhonde      Seq. Line : 14  
 Acq. Instrument : Instrument 1      Location : Vial 73  
 Injection Date : 12/10/2013 02:49      Log : 1  
    Inj Volume : 2.000 µl  
 Acq. Method : D:\DATA\MISC2 2013-12-09 19-54-27\MISC ANTI-BIOTICS\_00  
 Last changed : 12/9/2013 19:53 by Tungmiray Mhonde  
 Analysis Method : C:\CHEM5\AN\BIO\MS\MISC ANTI-BIOTICS QUANT.M  
 Last changed : 1/29/2014 09:54 by Zee  
    (modified after loading)  
 Method Info : Analysis of Antibiotic Residues in Milk

Additional Info : Peak(s) manually integrated



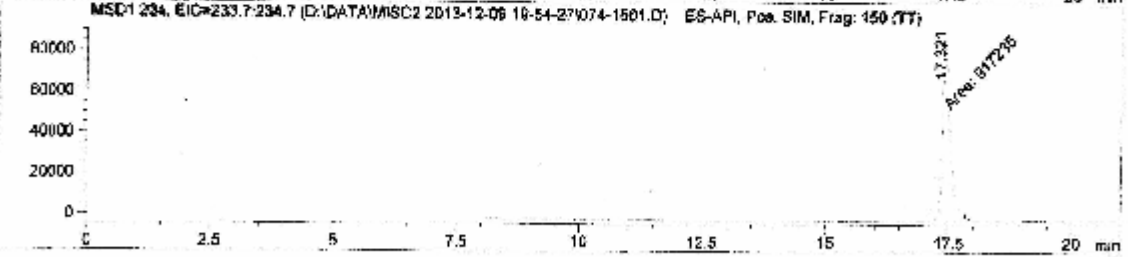
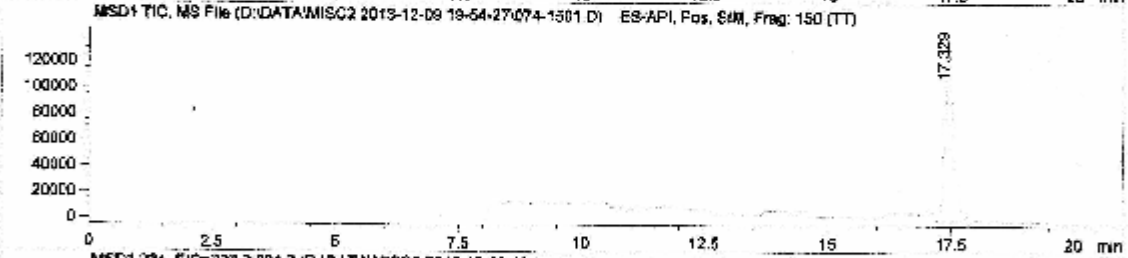
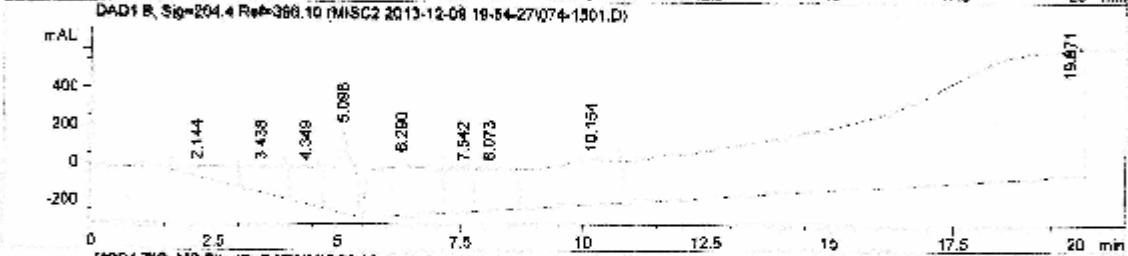
Data File D:\DATA\MISC2 2013-12-09 19-54-27\074-1501.D

Sample Name: Solv Cal Std 4

***ALBENDAZOLE CALIBRATOR 3 (STANDARD CONC - 1µg/ml)***

Acq. Operator : Tungamirayi Khanda                   Seq. Size : 15  
 Acq. Instrument : Instrument 1                        Location : Vial 74  
 Injection Date : 12/10/2013 03:21                   Inj : 1  
   Inj Volume : 2.000 µl  
 Acq. Method : E:\DATA\MISC2 2013-12-09 19-54-27\AMTSC\_ANTIBIOTICS\_QU  
 Last changed : 12/9/2013 19:53 by Tungamirayi Khanda  
 Analysis Method : C:\CHEM32\AMTSC\MSC\_ANTIBIOTICS\_QUANT.M  
 Last changed : 4/29/2014 09:34 by Zee  
   (modified after loading)  
 Method Info : Analysis of Antibiotic Residues in milk

Additional Info : Peak(s) manually integrated



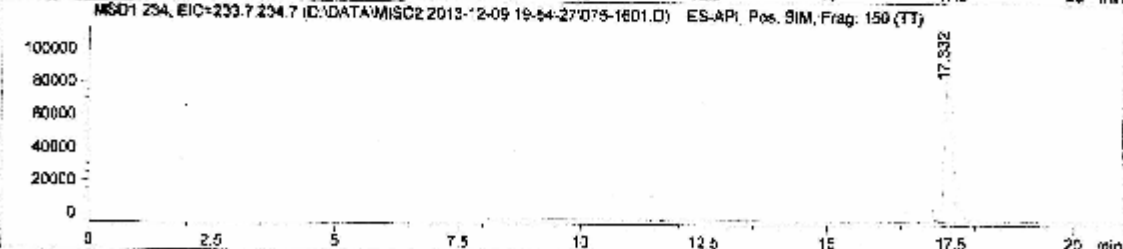
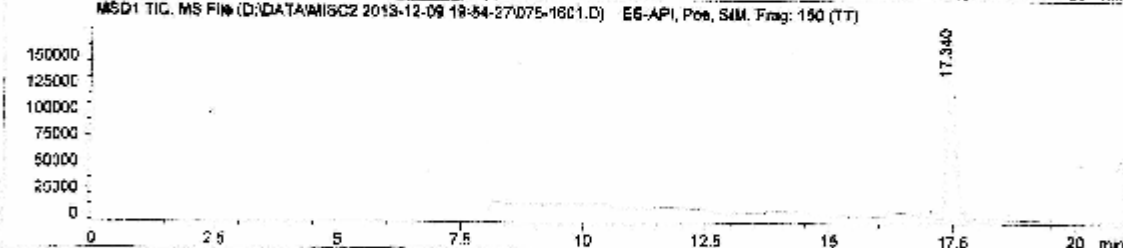
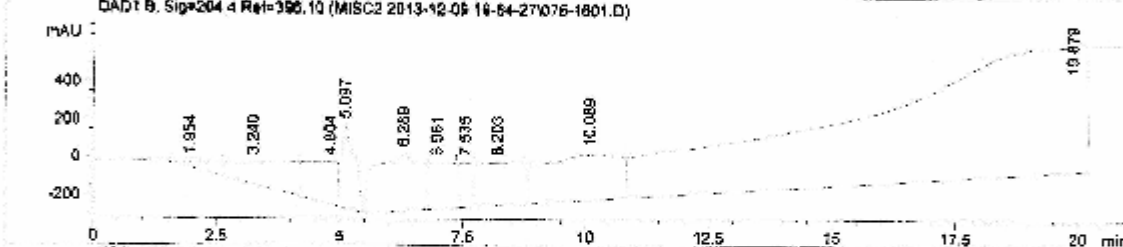
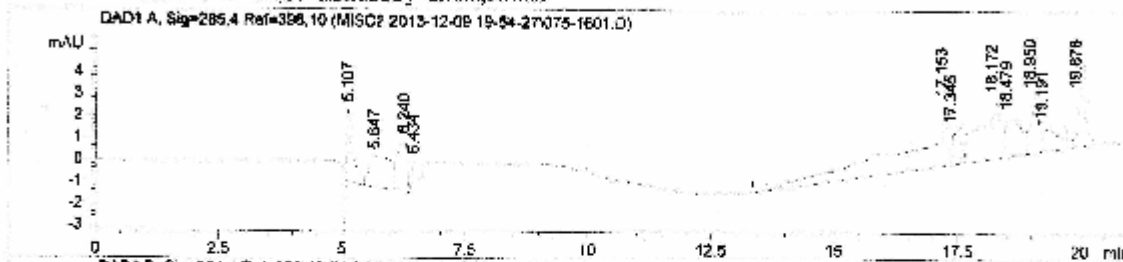
Sample Name: Sole Cal ADZ 5

*CLARENDAZOLE CALIBRATION 4 (STANDARD - CONC. 1.25 ug/ml)*

Acq. Operator : Tungamirayi Mhande      Run Time : 16  
 Acq. Instrument : Instrument 1      Injection : Vial 75  
 Injection Date : 12/10/2013 09:53      Inj : 1  
    Inj Volume : 2.000 ul

Acq. Method : D:\DATA\MISC2 2013-12-09 19-54-27\MISC\_ANTIBIOTICS\_QUANT.M  
 Last changed : 12/9/2013 19:53 by Tungamirayi Mhande  
 Analysis Method : C:\CHEM32\ANALMETHODS\MISC\_ANTIBIOTICS\_QUANT.M  
 Last changed : 4/29/2014 09:34 by Zec  
    (modified after loading)  
 Method Info : Analysis of Antibiotic Residues in milk

Additional Info : Peak(s) manually integrated



*Peak area on the last page.*

ALBENDAZOLE CALIBRATOR 5 (STANDARD - CONC 1.5 µg/ml)

Acq. Operator : Tungamirayi Mhande Seq. Line : 17  
 Acq. Instrument : Instrument 1 Location : Vial 96  
 Information Date : 12/10/2013 04:23 Inj : 1  
 Inj Volume : 2.000 µl  
 Acq. Method : D:\DATA\MISC2 2013-12-09 19-54-27\MISC\_ANTIBIOTICS\_Q  
 Last changed : 12/9/2013 19:33 by Tungamirayi Mhande  
 Analysis Method : C:\MSDCHEM321\METHODS\VTSC\_ANTIBIOTICS\_QUANTUM  
 Last changed : 4/29/2014 09:34 by Zoo  
 (modified after loading)  
 Method Info : Analysis of Antibiotic Residues in Milk

Additional Info : Peak(s) manually integrated

