INVESTIGATION OF THE PYHSICAL AND CHEMICAL PROPERTIES OF MILK CONTAINING ANTIBIOTICS

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ABSTRACT

INVESTIGATION OF THE PHYSICAL AND CHEMICAL PROPERTIES OF MILK CONTAINING ANTIBIOTICS

This work aimed to find a basic and rapid screening method for antibiotic residues in UHT whole cow's milk. For this purpose an investigation was conducted to screen some physical (e.g. acidity, pH, density, freezing point and electrical conductivity), thermo-physical (e.g. melting temperature, heat of fusion, evaporation temperature and heat of evaporation) and chemical properties (e.g. fat%, protein%, lactose%, minerals%, SNF%) of antibiotic free milk and milk fortified by Penicillin G, Ampicillin, Tetracycline. We can able determine whether residue of antibiotics making any difference on these selected properties. Thermo-physical properties were measured by differential scanning calorimeter (DSC), (TA Instruments, USA) and chemical properties were determined by using Lactostar (Funke Gerber Inc., Berlin, Germany).

Antibiotic residues were detected by Copan Milk Test, Penzyme Test and ROSA Test and by high performance liquid chromatography (HPLC) method for confirmation of screening tests. Due to some drawbacks of screening tests, liquid chromatography was required for confirmation of antibiotic residues in milk. HPLC method showed that average recoveries of spiked Penicillin G at 2, 4, 8 ppb, spiked Ampicillin at 2, 4, 8 ppb and spiked Tetracycline at 100, 250, 500 ppb were ranged from 44.67% to 66.00%, from 62.50% to 87.52% and from 92.86% to 94.35%, respectively.

We found that the acidity, pH and density of milk were independent of Penicillin G, Ampicillin and Tetracycline concentrations. Electrical conductivity (EC) were evaluated by applying ANOVA with Fisher's test and Probabilistic neural network (PNN) method. ANOVA was also performed for DSC and Lactostar measurement results. This evaluation suggested that EC measurement can be a great promising technique for detection of antibiotic residues in milk, DSC is a good characterization tool for understanding of thermal events and the presence of antibiotic residues in milk influencing freezing point and minerals (EMC)%.

ÖZET

ANTİBİYOTİKLİ SÜTLERİN FİZİKSEL VE KİMYASAL ÖZELLİKLERİNİN İNCELENMESİ

Bu çalışmada, UHT yağlı inek sütündeki antibiyotik kalıntılarının tespiti için basit ve hızlı bir metodun bulunması amaçlanmıştır. Bu amaçla, antibiyotik içermeyen ve Penisilin G, Ampisilin, Tetrasiklin içeren süt örneklerinin bazı fiziksel özelliklerinin (asitlik, pH, yoğunluk, donma noktası ve elektriksel iletkenlik), ısısal davranışlarının (erime sıcaklığı, erime ısısı, buharlaşma sıcaklığı ve buharlaşma ısısı) ve kimyasal (% yağ, % protein, % laktoz, % mineral, % yağsız kuru madde) özelliklerinin belirlenmesi için bir araştırma yürütülmüştür. Antibiyotik kalıntılarının seçilmiş bu özellikler üzerine herhangi bir etkisinin olup olmadığı tespit edilmiştir. Termal özellikler difransiyel taramalı kalorimetresi (DSC), (TA Instruments, Amerika Birleşik Devletleri), kimyasal özellikler ise Lactostar cihazı (Funke Gerber, Berlin, Almanya). kullanılarak ölçülmüştür.

Antibiyotik kalıntıları Copan, Penzyme, ROSA süt testleri ve bu hızlı testlerin doğrulaması için HPLC kullanılarak tespit edilmiştir. Hızlı testlerin bazı dezavantajları nedeniyle, sıvı kromatografisine sütteki antibiyotik kalıntılarının doğrulaması için gerek duyulmuştur. Doğrulama için HPLC ile elde edilen geri kazanım sonuçları Penisilin G için %44.67 - %66.00, Ampisilin için %62.50 - %87.52, Tetrasiklin için %92.86 - %94.35 değerleri arasında bulunmuştur.

Sütün asitliği, pHsı ve yoğunluğunun Penisilin G, Ampisilin ve Tetrasiklin konsantrasyonlarından bağımsız olduğu tespit edilmiştir. Elektriksel iletkenlik varyans analizi ve PNN methodu uygulanarak değerlendirilmiştir. DSC ve lactostar ölçüm sonuçlarına da varyans analizi uygulanmıştır. Değerlendirmeler, elektriksel iletkenlik ölçümünün sütte antibiyotik kalıntılarının tespiti için kullanılabileceğini, DSC'nin sütün ısısal davranışlarının daha iyi anlaşılması için iyi bir karakterizasyon aracı olabileceğini ve sütteki antibiyotik kalıntılarının varlığının sütün donma noktasını ve % mineral miktarını etkilediğini göstermiştir.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

Antibiotics are widely used in dairy cattle management for the treatment and the control of diseases, including mastitis, and also used as dietary supplements. The inadequate usage of antibiotics may results in drug residues passing into milk (Santos, et al. 2006). Antibiotic residues are undesirable in milk and milk products for a number of reasons. They can create a negative image of dairy and dairy products (McEwen, et al. 1991). They may cause allergic reactions in sensitive individuals and their exposure may lead to an increase in the numbers of resistant to antibiotics individuals. In addition, antibiotics interference with starter cultures for dairy products and influence negatively coagulation process (Rinken and Riik 2006).

The withholding period following treatment should not be forgotten to avoid antibiotic residue in milk by farmers. However, sometimes antibiotic residues can be contained in milk over withdrawal times, causing positive test results for antibiotic residues (Kang, et al. 2005). Antibiotic residues in milk are an increased risk. The frequent use of part-time employees, use of medicated feeds, failure to use separate equipment to milk treated cows and use of parlor milking systems are farm management factors that have been associated with this risk (McEwen, et al. 1991). To protect the consumer, the EU Maximum Residue Limits (MRLs) for veterinary medicinal products in milk have been established by the EU Council Regulation (EEC) No: 2377/90 (Zvirdauskiene and Salomsskiene 2007) and the residue levels of veterinary drugs in the raw and drinking milk must not exceed the limits stated in the 6th part of Turkish Food Codex Regulation No: 2002/30 (KKGM 2008).

Residue analysis includes both screening and confirmatory methods. Present methods for the detection of antibiotic residues involve microbial like microbial growth inhibitor tests, microbial receptor assays, enzymatic assays, immunologic assays or receptor-based methods and chemical-physical methods such as spectrophotometric, chromatographic, and fluorimetric methods (Le Breton, et al. 2007). The available microbiological tests are relatively slow and nonspecific for a one antibiotic, considering immunoassays that are usually quite expensive. Some of the screening methods are characterized as being rapid, with high throughput, being rugged,

inexpensive and sensitive but sometimes they give false negative or false positive results (Teagasc 2004). Disadvantage of the chemical-physical methods such as HPLC and mass spectroscopy contain complex steps, small amount of samples analyzed per time unit, and the need for trained personnel with high expertise (Rinken and Riik 2006). The best in residue analysis is for methods that have all the necessary quality attributes which can measure a wide range of veterinary drug residues directly in the food and which can produce results immediately and for a definitive nature. Therefore, a simple, fast, inexpensive and sensitive analytical procedure to detect antibiotic residues in milk is needed (Teagasc 2004). The development of biosensors has a significance potential to provide this demand. Biosensor gives results in less time. The preliminary preparation of the samples is not required. They have high selectivity and sensitivity properties (Rinken and Riik 2006).

The origin of idea of this work is based on an interest to find a basic and rapid method which is going to be a basis for development of a biosensor for detection of antibiotic residues in milk and milk products. For this purpose an investigation was conducted to screen some of the physical (e.g. acidity, pH, density, freezing point and electrical conductivity), thermo-physical (e.g. melting temperature, heat of fusion, evaporation temperature and heat of evaporation) and chemical properties (e.g. fat%, protein%, lactose%, minerals%, SNF%) of milk containing antibiotics in UHT whole cow's milk. This study also includes comparison of available methods for detection of antibiotic residues in milk and assessment of advantages and disadvantages of each method. For this purpose, Penicillin G (at concentrations of 2, 4, 8 ppb), Ampicillin (at concentrations of 2, 4, 8 ppb) and Tetracycline (at concentrations of 100, 250, 500 ppb) were chosen as a target antibiotics in this study. All of the measurements were conducted both on the whole milk containing these antibiotics and on whole milk free from antibiotics used as a control.

CHAPTER 2

A GENERAL VIEW OF ANTIBIOTIC USAGE

All of the health programs on television and newspapers have made the general public much more aware of food safety issues. Chemical and bacterial contamination of foods may happen at any phase of food production and may have harmful consequences for consumers. In other words, a trader can suffer great financial losses or maybe remove if his products are found to be contaminated (Hall, et al. 2003).

The development of an extent of agrochemical and veterinary drugs, drawing attention of agricultural production and increase in industrialization are considered environmental contamination points with respect to an increased exposure of the consumer to chemical residues from food and other sources (O'Keeffe and Kennedy 1998). The consumer looks increasingly for "pure" food. In regard to residues in food, the attention is increasingly towards natural or "organic" systems for food production (O'Keeffe and Kennedy 1998).

Antibiotics are molecules that stop microbes (both bacteria and fungi) from growing or killing them (Al-Jabri 2005). Generally, antibiotics have been used by farmers and veterinarians to control and treat of infectious diseases of dairy cattle (Albright, et al. 1961), by aiding in prevention of diseases and by enhancing the performance of animals maintained in production agriculture. The major benefits resulting from the use of low levels of antibiotics in animal production are economic. Antibiotics are used in this way to increase the rate of weight gain and/or improve feed efficiency in cattle breeding (Gustafson and Bowen 1997).

Mastitis was the first disease of dairy cattle to be treated with antibiotics (Ruegg 2005, Milner, et al. 1997). Antibiotics are implemented to dairy cattle by the way of several routes: (1) infusion into the udder for the treatment of mastitis, (2) injection for the treatment of numerous diseases and (3) orally for treatment or prevention of diseases or as a dietary supplement. Antibiotics in milk are mainly the result of improper use of mastitis infusion preparations, or of failure to confirm to the instructions on label, to the effect that milk from treated quarters be discarded or used for purposes other than human consumption (Albright, et al. 1961).

2.1. Benefits and Risks of Antibiotics Usage

Antibiotic residues are undesirable in milk and milk products for public health reasons and because of their potential impact on manufacturing process (Ruegg and Tabone 2000, Yamaki, et al. 2004). They can create a negative image of dairy and dairy products in the eyes of the public (McEwen, et al. 1991, Ruegg and Tabone 2000). The presence of antibiotic residues in milk was considered primarily a manufacturing problem related to inhibition of dairy starter microorganisms and cause economic losses to the cheese and fermented milk industries (Al-Jabri 2005, Kang'ethe, et al. 2005). Hence, it would be regarded in many countries as 'adulterated' and a public health risk, if the milk is to be processed for direct retail sale or into milk powder or some other product (Yamani, et al. 1999).

The presence of antibiotics in milk has been prohibited, due to the fact that they are sometimes associated with adverse effects on host which comprise hypersensitivity, depletion of beneficial gut and mucosal microorganisms, immunosuppression and allergic reactions (Al-Jabri 2005). Penicillin in very small concentrations found in milk may cause allergic reactions in highly sensitive individuals (Albright, et al. 1961, Chenh Chen and Chain Chang 1994) after ingestion, including skin rashes, asthma, anaphylactic shock and even death (Yamani, et al. 1999). Minute amounts of drug residues can be carcinogenic, teratogenic, mutagenic cause enzyme induction and inhibition and interact with other environmental chemicals (Seymour, et al. 1988). Beforehand, certain antibiotics used in animal farming are now prohibited for use in food-producing animals; chloramphenicol, because of its potential toxicity for sensitive humans, and the nitrofurans, because of their mutagenic, carcinogenic and bound-residue characteristics (O'Keeffe and Kennedy 1998).

Furthermore, any exposure of the intestinal micro flora of humans to antibiotics may lead to an increase in the numbers of antibiotic-resistant species present (Yamani, et al. 1999, Adesiyun and Webb 1997). If some of these are pathogenic, they could have dreadful consequences (Yamani, et al. 1999). Fermented milk products use lactic starter cultures, and these bacteria enter into our intestines in large numbers where they interact with the intestinal micro flora. Commercial introduction of probiotics including antibiotic resistance strains may also have negative consequences, for example, when resistance is transferred to intestinal pathogens (Mathur and Singh 2005). Some

antibiotics are directly toxic, e.g. chroramphenicol which destroys blood-forming tissue. Allergic reactions and toxic side effects may have fatal results (Hall, et al. 2003).

Dairy manufacturing companies which are more directly affected than others by the presence of antibiotics in milk are those that produce fermented milk products. Cheese production is depending on lactate fermentation. All bacterial organisms which are included in the production of fermented milk products show varying degrees of inhibition of growth in the presence of the different antibiotics. Cheese manufacture is dependent on the rate of acid development as well as the total amount produced. If either rate or total quantity of acid is reduced from the optimum, the quality of cheese suffers. Active starter cultures are the key to successful manufacture of fermented dairy products. If they are inoculated into milk which contains traces of antibiotic residues, suitable or active cultures can not be maintained. Such contaminated milk constitutes a great economic risk. Producer would be required to withhold when following withholding recommendations. If it is used in their manufacture, milk which contains antibiotic residues will contaminate other dairy products. If the milk is dried, evaporated, or made into ice cream, the antibiotics is concentrated in these products. Despite the fact that no manufacturing problems result from the presence of antibiotics in the above-mentioned products, a consumption or use problem is obvious (Albright, et al. 1961).

2.2. Factors Influencing the Occurrences of Antibiotic Residues in Milk

Antibiotic residues pass into the milk supply at the farm level and milk producers themselves bear the final responsibility for selling antibiotic residue free milk. It is important that producers understand the factors that lead to antibiotic residues in milk and how these residues can be prevented. A survey of farms in the United Kingdom was conducted by Booth and Harding, the three most common reasons for residue occurrences suggest by farmers were failure to withhold milk for the proper length of time, accidental transfer of milk from treated cows to bulk tanks, and prolonged excretion of drug from treated cows. A mail survey of Michigan farmers with positive and with negative bulk milk antibiotic residue tests was conducted by Kaneene and Ahl. Milk residues were associated with each of the following: increasing frequency of use of medicated feed, herd size, and numbers of hired persons. Farmers in the

Michigan study thought that the most important management factors leading to drug residues were insufficient knowledge about drug withdrawal periods, errors due to hired help, insufficient records of treatment and identification of animals (McEwen, et al. 1991, Jones 1999).

Researches have indicated that farmers sometimes forget to withhold milk from treated cows for the proper time, but other mistakes, such as withholding of milk only from treated quarters while placing milk from untreated quarters into the bulk tank, have also been described. Farm management factors that have been associated with an increased risk of residues in milk involve the frequent use of part-time employees, use of medicated feeds, use of parlor milking systems, and failure to use separate equipment to milk treated cows (McEwen, et al. 1991, Gustafson 1991).

2.3. Regulatory Controls Antibiotic Residues in Milk

Milk producers must guarantee their milk. It must not contaminated by any veterinary drugs from the list of prohibited antimicrobials or the levels of these materials are lower than the Maximum Residue Limits (MRLs) (Zvirdauskiene and Salomsskiene 2006). MRLs are considered that the drug may be safely used without harming the consumer (Hall, et al. 2003).

There are two interpretations of residues in food. These are Maximum Residue Limits (MRLs) and Acceptable Daily Intake (ADI) values. The MRL is the maximum concentration of a residue. It was expressed as mg per kg food, legally permitted in or on food commodities and animal feeds. MRL values for a residue are particular to each food or food type. The Acceptable Daily Intake (ADI) value is an estimate of the amount of residue. It was expressed as mg per kg body weight that can be ingested daily over a lifetime without appreciable health risk. The ADI is based on a toxicological evaluation, under a range criteria, of the chemical and is based on the no-adverse-effect level in test animals and contains safety factors to account for inter-species differences (normally x 10) and differences between humans (normally x 10), such as vulnerable (sick) individuals, infants, elderly, etc (O'Keeffe and Kennedy 1998).

The Codex Alimentarius Commission of the Food and Agriculture Organization (FAO) and World Health Organization (WHO) of the United Nations stated in 2001 that the scientific literature for effects of processing on drug residues in milk is insufficient

to permit clear determination of the effect, if only, that processing may have on the level of most drug residues that could occur in milk and that additional studies are needed in this area (Grunwald and Petz 2003).

The EU Maximum Residue Limits (MRLs) for veterinary medicinal products in milk were established by Council Regulation (EEC) No.2377/90 (Zvirdauskiene and Salomsskiene 2006). The residue levels of veterinary drugs in the raw and drinking milk must not exceed the limits stated in the 6th part of Turkish Food Codex Regulation No:2000/6 (ABGS 2008), (Table 2.1).

Table 2.1. MRLs at EU Codex and at Turkish Food Codex
(Source: Copan Sciences Literature 2008)

Antimicrobial Agents	EU / Codex MRL[1] (ppb)	Turkish Food Codex MRL (ppb)
Beta-lactams		
Penicilin G	4	4
Ampicillin	4	4
Amoxicillin	4	4
Cloxacillin	30	30
Dicloxacillin	30	30
Oxacillin	30	30
Naficillin	30	30
Ceftiofur[2]	100[3]	100
Cefquinom[7]	20	20
Cefapirin	10	10
Cefoperazon	50	50
Cefalexin	100	100
Cefazolin	50	50
Tetracyclines		
Chlortetracycline[2]	100[4]	100
Oxytetracycline[2]	100[4]	100

(Cont. on next page)

7

Table 2.1 (cont.). MRLs at EU Codex and at Turkish Food Codex
(Source: Copan Sciences Literature 2008)

Tetracycline[2]	100[4]	100
Doxycycline[2]	100[4]	100
Sulphonamides		
Sulfathiazol	100[6]	100
Sulfamethazine[5]	100[6]	100
Sulfadioxine	100[6]	100
Sulfadimethoxin	100[6]	100
Sulfadiazin	100[6]	100
Sulfamethoxazole	100[6]	100
Sulfamonometossina	100[6]	100
Aminoglycosides		
DH-Streptomycin	200	200
Streptomycin	200	200
Neomycin	500	500
Gentamicin	100	100
Spectinomycin	200	200
Macrolides		
Erythromycin	40	40
Spiramycin	200	200
Tylosin	50	50
Tylmicosin	50	50
Other antibiotics		
Dapson	0[7]	0
Trimethoprim	50	50
Tiamfenicol	50	50
Chloramphenicol	0[7]	0

1. Regulation 2377/90 ff EEC   5. Sulfadimidine

2. Mother compound   6. Sum of all substances of this group

3. Mother compound and metabolites   7. Not allowed

4. Mother compound and 4-epimer

2.4. Classes of Antibiotics

Some of the more important classes of veterinary drugs are sulphonamides, β-lactams (e.g. penicillin), tetracyclines, aminoglycosides (e.g. streptomycin), macrolids (e.g. erythromycin), peptide antibiotics (e.g.virginiamycin) and ionophores (e.g. monensin) (O'Keeffe and Kennedy 1998).

Beta-lactams (combined total beta-lactams and cloxacillin, 70), followed by tetracyclines (40) and gentamycin/neomycin-type aminoglycosides (40) were recorded by means of analysis, in other words, positive results (Figure 2.1).
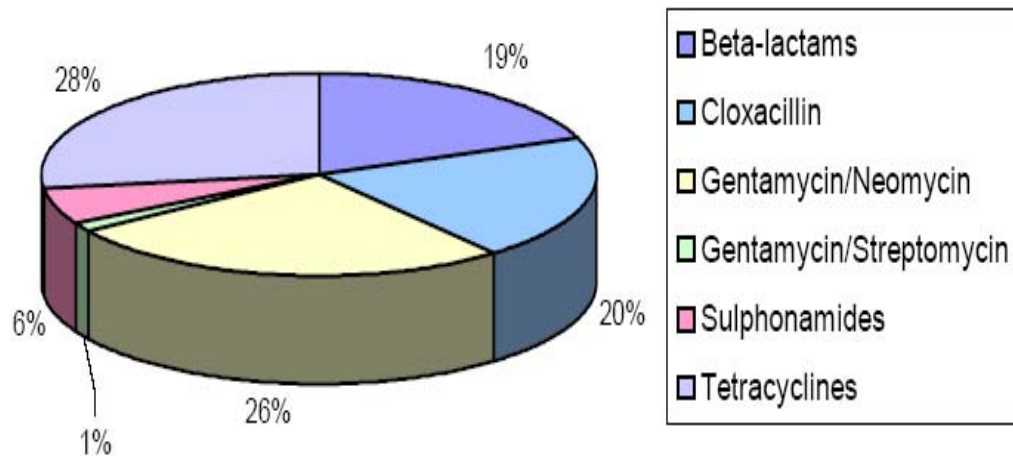
Figure 2.1. Antibiotic residues detected in milk 1997-2003 by percentage

(Source: Hall, et al. 2003)

2.4.1. Beta-Lactam Antibiotics

Antibiotics of the beta-lactam group are extensively used for treatment of bacterial infections. They are the preferred drugs for the treatment of clinical mastitis in dairy cows. In a corresponding manner, beta-lactams comprise the major source of antibiotic residues in milk (Lamar and Petz 2007).

The beta-lactam antibiotics (namely Penicillin-G, Amoxicillin, Ampicillin and Cloxacillin), also known as penicillin, are widely used in veterinary medicine. The

presence of beta-lactam residues in food may be responsible for allergic reactions in sensitive individuals. The beta-lactam ring system, a highly strained and reactive cyclic amide featured by these antibiotics. The beta-lactam ring system makes them susceptible to a variety of degradative processes. Reaction with hydroxide ion opens up the beta-lactam ring to produce an inactive compound. Also, beta-lactams are acid sensitive, and degrade at low pH by a more complex mechanism. Alcoholic solutions of these antibiotics are therefore unstable due to the acidic character of alcohols. Polarity is highly dependent on the nature of the group attached to the ring (Santos, et al. 2006).

Penicillins are a class of beta-lactam antibiotics and also a group of antibacterial compounds inhibiting bacterial cell wall synthesis. They are highly sensitive to heat, acids and penicillinases. The degradation of penicillins is affected by different factors like temperature, pH, ionic strength, metal ions, degree of crystallinity, solvent composition (Michnik, et al. 2004).

In addition, penicillins are one of the oldest groups of antibiotics. They still use extensive clinical utility. One major area is the treatment of bovine mastitis which causes in Germany a yearly economic damage of about € 1 billion per year according to an estimation of the German Veterinary Society. Penicillins are not inherently very toxic. However, they can produce strong allergic reaction in sensitized humans and concentrations above the MRL inhibit bacteria used in the fermentation process employed by the dairy industry. Little, however, is known about the effect food technological processing on the destruction of penicillin residues in milk or other matrices (Grunwald and Petz 2003).

The name "penicillin" can also be used in reference to a specific member of the penicillin group. All penicillins possess the basic Penam Skeleton, which has the molecular formula $R-C_9H_{11}N_2O_4S$, where R is a variable side chain (Figure 2.2), (Ashnagar and Gharib 2007).

Ampicillin is a beta-lactam antibiotic that has been used widely for treatment of bacterial infections since 1961. It can sometimes cause allergic reactions. Ampicillin, belonging to the group of beta-lactam antibiotics, is able to penetrate gram-positive and some gram-negative bacteria (Ashnagar and Gharib 2007).
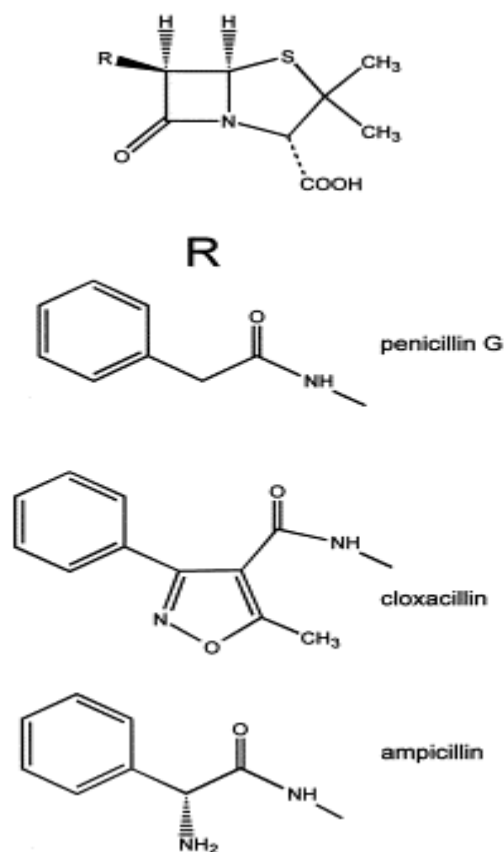
Figure 2.2. Structure of some beta-lactams, Penicillin G and Ampicillin

(Source: Kennedy, et al. 1998)

2.4.2. Tetracyclines

Tetracyclines are broad-spectrum antibiotics, such as tetracycline (TC), chlortetracycline (CTC), doxycycline (DTC), and oxytetracycline (OTC), (Charoenraks T. 2005). Tetracyclines have been used for more than 50 years for the treatment of mastitis and are added to cattle feeds to increase growth rates (Santos, et al. 2006).

These uses have the potential to result in the presence of tetracycline residues in milk, if these antibiotics have been improperly administrated or if the withdrawal time for the treated cows has not been observed. Tetracycline residues in milk may stimulate harmful effects on humans, such as allergic symptoms, liver damage, yellowing of teeth, and gastrointestinal disturbance because of the selective pressure of antibiotics on human gut micro flora or may lead to financial losses in the dairy industry by inhibiting starter cultures in food technological processes (Reid, et al. 2006). Moreover, trace

amounts of antibiotic residues in milk favor the development of antibiotic-resistant bacteria (Fritz and Zuo 2007).

Figure 2.3 shows the chemical structures of tetracycline. However not only the concentration of tetracycline residues but also their degradation products, in animal fluids and tissues are significant in understanding the potential effects of tetracycline antibiotics on human and animal health (Fritz and Zuo 2007).
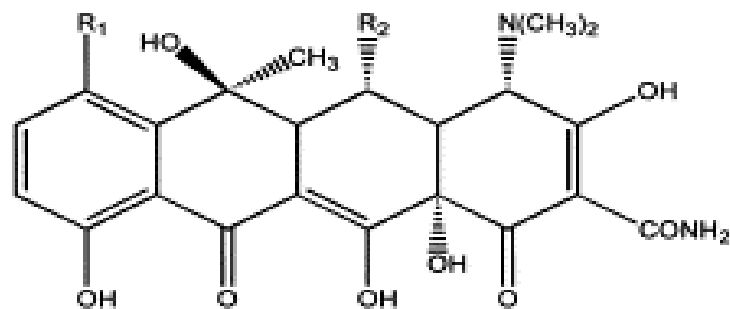
Compound	R_1	R_2
Oxytetracycline	H	OH
Tetracycline	H	H
Chlortetracycline	Cl	H

Figure 2.3. Structure of tetracycline

(Source: Kennedy, et al. 1998)

2.5. Reducing the Risk for Antibiotic Contamination of Milk

It is significant for dairy producers and dairy veterinarians to understand the terms and fundamentals concern related to antibiotic use in dairy cattle. Discussion of these factors will help the dairy industry take a leading role in making responsible antibiotic use decisions and laws for dairy cattle (Callan 2000).

Definite precautions, such as the use of residue test kits, using separate milking equipment, taking special care when part-time employees must be used, and increasing farmer knowledge of drug residues, can decrease the possibility of residues occurrence (McEwen, et al. 1991).

The HACCP program for antibiotic avoidance, the Milk and Dairy Beef Quality Assurance Program (MDBQAP), has been developed in cooperation by the American Veterinary Medical Association and the National Milk Producers Federation. The program defines 10 critical control points for preventing antibiotic residues. These 10 points concentrate initially on disease prevention and management of antibiotics and treated cows, in spite of the fact that one point is to use screening tests for drug residues. The producer manual for the MDBQAP suggests that simply following label recommendations on milk withholding times ensures a safe product; yet, when antibiotics are used in an extra label manner, screening tests for drug residues should be used if such tests are available (Sischo 1996).

The use of antibiotic residue screening tests and implementation of good management practices on dairy farms have been positively correlated with reductions in the occurrence of antibiotic residues in milk. In recent times, for a reduction in the risk of residue violations, antibiotics residue screening tests for evaluating individual cow's milk have been used. Additionally, the Milk and Dairy Beef Residue Prevention Protocol of the Dairy Quality Assurance Program suggests that milk from individual cows be tested for antibiotic residues following extra-label use of an antibiotic. Testing milk from antibiotic-treated cows following an appropriate milk-withholding period allows the dairy producer to make informed decisions about milk withholding and reduces the risk of antibiotic contamination of commingled milk (Andrew 2000).

Several surveys have reported that false-positive results occurred on samples containing no antibiotic residues in the tests. High levels of natural inhibitors are present in mastitic milk and in colostrums. They can cause false-positive results in the microbial growth inhibition assays. Drugs are widely used in the treatment of various bacterial infections, including mastitis. The recommended withholding period following treatment should be followed to avoid drug residue in milk. However, sometimes antibiotic residues can be not only involved in milk over withdrawal times but also resulting in positive test results for antibiotic residues. Besides, natural inhibitors in the milk of cows with mastitis are increased and kept at high concentrations for several days. The increased natural inhibitors can cause false-positive results in the use of bioassays based on bacterial growth inhibition on the milk samples over withdrawal times. Hence, it is important to evaluate that the positive results in milk over withdrawal times are lead to by drug residues or natural inhibitors (Kang, et al. 2005).

If farmers and processors are educated about the potential hazards associated with antibiotic residues in foods of animal origin, antibiotic residues can be prevented from entering the food chain at the producer level. Processors and producers should also be made aware of the financial losses. There are now several programs in the USA and Europe which make use of HACCP principles in quality management systems for farms, e.g. Milk & Dairy Beef Quality Assurance Program Milk and Dairy Beef Residue Prevention Protocol. In this program, the farmer works closely with a veterinarian and follows a 10-step process to minimize the risk of antibiotic residues entering the food chain (Hall, et al. 2003).

The Food and Drug Administration has begun a three-phase educational program to eliminate antibiotics from the milk supply: (1) education of the dairy farmer, educational programs participated in by veterinarians, dairy inspectors and sanitarians, dairy school, and government can be helpful, but especially dairymen (2) issuance of a warning statement (3) reduction in concentration of antibiotic in treatment of mastitis (Albright, et al. 1961).

Restricted sales of antibiotics for treatment and control of mastitis might reduce antibiotic contamination of milk. In identification of this problem, English authorities recently restricted antibiotics for animal use to veterinarians only. Penicillin, chlortetracycline, oxytetracycline, streptomycin, and chloramphenicol in milk are relatively stable to pasteurization temperatures and above. It would be desirous to know the effects of ultra-high heat treatment of milk for relatively short periods of time on antibiotics present in milk. When antibiotics were kept at low temperatures (0 to 10 ° F) for periods up to 12 wk., the property of the antibiotic to prevent normal growth of lactic starter culture was not changed. Milk from treated cows when added to the central milk supply would be diluted to the extent that there would not be any difficulty for consumption purposes or in the manufacture of cultured products. If whole milk containing antibiotics is separated into cream and skim milk components, concentrations of either penicillin or streptomycin are equal in the two fractions. Several substances have been found to inactivate penicillin. One of these is hydroxylamine. It was effective but could not be advised for practical purposes, because it is slight toxicity. The taka-diastase and amylase, both derived from an *Aspergillus* strain, were able to inhibit penicillin activity (Albright, et al. 1961).

CHAPTER 3

IDENTIFICATION AND QUANTIFICATION OF

ANTIBIOTIC RESIDUES IN MILK

3.1. Introduction

The objective of this chapter includes comparison of available methods for detection of antibiotic residues in milk and assessment of advantages and disadvantages of each method.

Antibiotic residues determination in the food is significant to be sure the quality and safety of the foodstuff. There are a number of different techniques for detection of antibiotic residues in milk (Molina, et al. 2003). Generally, antibiotic residue analysis contains not only screening but also confirmatory methods. For screening, several commercially available test kits are marketed for antibiotic residue detection in milk. They neither are nor equal in terms of lowest detection limit, repeatability or specificity. The screening methods are inhibitory tests, receptor assays or immunoassays and confirmatory methods, such as chromatography with UV, fluorescence or mass spectrometry detection (Le Breton, et al. 2006, Setford, et al. 1999). Rapid screening tests are widely used to detect the presence of antibiotic residues in milk but more accurate chromatographic methods are required by government regulatory agencies to identify and confirm identity and quantity of antibiotic residue present. On the other hand, in spite of the advantages, there are some drawbacks of screening tests. They can not determine which antibiotics are present in the milk. The outcomes of many of these tests are false-positive or false-negative. For instance, the presence of high somatic cell counts results in false positives. In contrast to chromatographic methods, they may detect antibiotic residues at levels far below the safe levels (Schenck and Callery 1998). False-positive test kit results might lead to unjustified waste of milk and several economic losses. The data on a rate of true false-positive tests or data on how much milk was discarded because of false-positive test results was not been able to found.

This case can affect the dairy industry negatively. Since false-positive results create negative image among consumers, producers, veterinarians and regulatory personnel because of screening inadequately for safety (Coffman, et al. 1999).

For these reasons, more sensitive and specific analytical methods are required. Most commonly, liquid chromatography (LC) and gas chromatography (GC) are the techniques used (Schenck and Callery 1998). These methods are extremely sensitive and validate the presence of antibiotic residue structure and also their concentrations (Coffman, et al. 1999). Costly in time, equipments and chemicals are drawbacks of the full procedure and the methodologies for confirmatory analysis. Besides, they require trained personnel with high expertise. A large number of samples, with a variety of analysts, to be analyzed in relatively short periods of time must be conducted by control laboratories. Thus, there is a requirement for screening methods that allow the analysis of such a large number of samples in short periods of time. In other words, high through-put methods with low cost must be available. These methods must be able to detect class of analysts at the level of interest (Van Peteghem, et al. 2001). The main requirements for a screening method are displayed in Table 3.1.

Table 3.1. Main requirements for screening methods
(Source: Toldra and Reig 2006)

Requirements
Easy to use
Low set-up costs
High through-put
Reduced time and low running costs for results
Sensitivity (no positives are lost)
Specificity (minimum number of false positives)
Repeatability

Another screening method is the immunological methods involve in ELISA test kits. There are several commercial kits available. Other immunological methods are depended on radioimmunoassay. Moreover, biosensors associated with immunological methods are available. There are two types of chromatographic methods. These are

HPTLC and HPLC. They combine with different detection systems. Different methods available for the screening of residues in animal foods are summarized in Table 3.2.

Table 3.2. List of main methods available for screening
(Source: Toldra and Reig 2006)

Inhibitor Tests	Immunological Methods	Chromatography Methods
Microbial inhibitor tests	ELISA test kits	HPTLC
Rapid tests	Radioimmunoassay	HPLC
	Multiarray biosensors	LC
		GC

It is concluded that most of the available microbiological methods are relatively slow and nonspecific. On the other hand, immunoassays are usually quite expensive. Among the screening methods, some of them are characterized as being rapid, with high throughput, being rugged, inexpensive and sensitive but they give false negative or false positive results. The chemical-physical methods including HPLC and mass spectroscopy require complex steps, small amount of samples being analyzed per time unit, and trained personnel with high expertise.

3.2. Rapid Screening Methods for Antibiotic Residues

Antibiotics are significant for control of mastitis and other diseases in dairy cattle. The presence of residues can cause number of problems. Maximum residue limits (MRLs) have been established by the European Community. For this reason, efficient detection methods of antibiotic residue are required (Le Breton, et al. 2007).

Several different methods are available for detection of antibiotic residues in milk. Inhibitory tests, receptor assays or immunoassays are widely used rapid screening methods for antibiotic residues (Le Breton, et al. 2007, Andrew, et al. 1997). Rapid tests were designed completing the test in short time for milk manufactures. These methods are simple. The most commonly available tests are microbial inhibitor tests with spores *Bacillus stearothermophilus* var. *calidolactis* Delvotest SP (DSM, Netherlands), Copan Test (Copan, Italy), Charm Farm-960 Test (Charm Sciences, Inc., USA); with

Streptococcus thermophilus-Valio T 101-test, Valio T 102-test (Valio, Finland); enzymatic tests- Penzyme, Penzyme S (UCB Bioproducts, Belgium); immunological tests- Delvo-X-Press β-Lactam (DSM, Netherlands), β-STAR (USB Bioproducts, Belgium), ROSA test (Charm Sciences, Inc.,USA). The brief scheme of the inhibitor tests is presented in Table 3.3.

Table 3.3. Milk tests for determining for antibiotic residues
(Source: Zvirdauskiene and Salomskiene 2007)

Type of Test	Test	Producer	Principle of Method	Inhibitors detected and sensitivity
Microbial inhibitor tests	Delvotest SP	DSM, Netherlands	Microbiological method with *Bacillus stearothermophilus* var. *calidolactis* C 953 spores	Penicillin G 0.003-0.004IU /ml, ampicillin 0.003-0.005 ,sulfamethazine 0.1-0.2 µg/ ml and others
	LPT	State Laboratory For Milk Control Lithuania	Microbiological method with *B. stearothermophilus* var. calidolactis C 953 spores	Penicillin G 0.004±0.001 IU/ml, sulfamethazine not < 1µg/ml, dapsone not < 0.003 µg/ml
	MaI-1	KTU Food Inst. Lithuania	Microbiological method with *B. stearothermophilus* var. calidolactis C 953 spores	Penicillin G 0.004±0.001 µg/ml, sulfamethazine not < 1µg/ml, dapsone not < 0.003 µg/ml
	Copan Single Test P&S 100	Copan, Italy	Microbiological method with *B. stearothermophilus* var. calidolactis C 953 spores	Penicillin G 0.002±0.001 µg/ml, sulfamethazine 0.15 ±0.05µg/ml,dapsone 0.003 ±0.001 µg/ml and others
	Valio T 101 Test	Valio, Finland	Microbiological method with *Streptococcus thermophilus*	Penicillin G 0.004+0.001 IU/ml, tetracyline-more than 0.2+0.1 µg/ml, sulfadimidine 1±0.5 µg/ml and others
Rapid tests	Delvo-X -PREES β-II	DSM, Netherlands	Receptor-ezyme assay	Penicillin G 0.002 µg/ml; ampicillin 0.004 µg/ml, amoxicyllin 0.004 µg/ml and others
	SNAP test	IDEXX Lab. Inc. USA	Enzyme immunoassay	Penicillin G 0.004 µg/ml; ampicillin 0.004 µg/ml, amoxicyllin 0.004 µg/ml and others
	ROSA test	Charm Sci. Inc., USA	Receptor assay	Penicillin G 0.004 µg/ml and others
	Penzyme S	UCB Bioproducts Belgium	Enzymatic method	Penicillin G 0.005-0.006 IU/ml
	β-STAR	UCB	Immuno/receptor assay	Penicillin G 0.003-0.001

The tests were evaluated in terms of the test procedures, the shelf life of the test, ability for use at laboratories and other features of the tests. These features of tests were established by EN ISO 13969:2004 [(Milk and milk products-Guidelines for a standardized description of microbial inhibitor tests (ISO 13969:2003)] and EN ISO 18330:2004 [(Milk and milk products-Guidelines for the description of immunoassays or receptor assays for the detection of antimicrobial residues (ISO 18330:2003)]. The short and brief evaluation of the test procedures is displayed on Table 3.4.

Table 3.4. Comparison of different tests according to usage

(Source: Zvirdauskiene and Salomskiene 2007)

Type of test	Test	Incubation temperature	Incubation time	Notes
Microbial inhibitor Tests	LPT	63.5 °C ± 0.5 °C	4 h 15 min- 4 h 30 min	One multiple for 96 samples. Duration of test is long but test is sensitive for many groups of inhibitory substance. Short shelf life 5 days from the date of manufacture. Suitable for screening of milk in a big laboratory
	MaI-1	63.5 °C ± 0.5 °C	4 h 15 min- 4 h 30 min	One test tube for 1 sample. Duration of test is long but test is sensitive for many groups of antibacterial substances. Shelf life 3 months from the date of manufacture. Suitable for single samples.
	Copan	64.5 °C ± 0.5 °C	3 h	Test is sensitive for some groups of antibacterial substance but for the smaller number of them than LPT and MaI-1. It is simple to use and read the result. Suitable for single samples. Shelf life 12 months.
	Valio T 101	42 °C ± 1 °C	4 h 30 min	It is necessary to heat milk for 5 Min at 92°C ± 2 °C before testing. The heating takes an additional time.
Rapid tests	Penzym S	47 °C ± 0.5 °C	25 min	Reagent No.1 is colourless so after adding 10 µl of it into eppendorf type vial it is diffucult to catch sight of it in the vial. It takes time to divide the tablets of Reagent 2 into the vials. The reading of results should be performed quickly. This method is not suitable for testing a large number of samples (>10) at once.
	SNAP	45 °C ± 5 °C	10 min	It is important to press the activator at the proper moment. The test is appropriate for a small number (2-4) of samples. It can be diffucult to read the results because of the similarity of the control and test sample spots.
	ROSA	56 °C ± 1 °C	8 min	The use of ROSA reader is recommended because, without it, it can be diffucult to determine which strip (test or control) is more intense.

3.2.1. Bacterial Growth Inhibition Methods

Bacterial growth inhibition method is one of the detection methods for antibiotic residue in milk. In the early 1940s, *Bacillus subtilis* was the target organism but nowadays methods have been developed that based on *Bacillus stearothermophilus* inhibition. Bacterial growth inhibition can be determined qualitatively and quantitatively. Generally, these method have been used for beta-lactams especially penicillin. For quantitative determinations of microbial inhibition methods; after incubation for the appropriate time, zones are measured. Firstly, zone of definite amount of penicillin is determined and then compared the sample zones. If the presence of penicillin wants to be confirmed, penicillinase (beta-lactamase), is an enzyme that inactivates penicillin specifically, is added to the sample (Hui 1993).

In the qualitative *B. stearothermophilus* var. *calidolactis* disc assay method, there is reference plate. This reference gives a definite zone of inhibition. All of the plates are incubated at appropriate temperature and time. According to the zone size, the presence of penicillin is determined. No zone of inhibition around the sample means beta-lactam negative (Hui 1993).

In addition, commercial methods are available. During growth, *B. stearothermophilus* var. *calidolactis* produces acid. Indicator dye changes in the absence of antibiotic residues. If the bacteria do not grow and produce acid, colour change can not be observed. This means inhibitors are present (Hui 1993).

There are lots of microbial inhibitor tests produced by several companies. These are the Brilliant Black Reduction Test, the Valio T101 test, the Copan microbial inhibitor test, Delvotest SP-NT, MaI-1, the LPT, ROSA test, β-STAR, the Lumac rapid antibiotic test, the Arla micro test and Biosys bioluminescence method. However, each of these tests is not available in all of the countries. Countries are not interested in all of them (Neaves 1999). Copan Milk Test and Delvotest SP-NT are new developed inhibition assays. Delvotest SP-NT is related to Delvotest SP and Delvotest Milk Control Stations (MCS) but with nutrients pre-incorporated in the agar. A nutrient table is not required. The Copan Milk Test has also the nutrient pre-incorporated in the agar (Le Breton, et al. 2007).

3.2.2. Competitive Binding Methods

Charm Sciences, Inc. (Malden, MA) has developed various test methods for detection inhibitory substances in milk. In this test, ^{14}C-labeled antibiotic and *Bacillus stearothermophilus* cells are combined with the sample. It competes for binding sites on the bacterial cell wall and more ^{14}C-label is free in solution, if antibiotic is present in the sample. The labeled antibiotic binds with the cell wall and is removed from the solution with centrifugation. This indicates that no antibiotic is present in the sample. Positive and negative controls are prepared. The results are compared to the controls (Hui 1993).

Charm II procedure has been widely used by many dairy laboratories. This screening method is used for several families of antimicrobial drugs. In this procedure, there are two different microorganisms. These microorganisms provide necessary binding sites. Then the labeled compound is detected. Moreover, different tests are designed for farm and small dairy plant by Charm Sciences, Inc. These tests can be easily utilized (Hui 1993).

Other competitive binding techniques are Penzyme and Penzyme III methods. The methods consist of the binding of DD-carboxypeptidase to beta-lactam antibiotics. Enzyme and sample are incubated at appropriate temperature and time. The substrate is added. The mixture is incubated at same temperature. A yellow color displays an antibiotic residue is present. A pink color means negative result. An orange/yellow color indicates the possibility of beta-lactam residues and so the sample should be confirmed. Positive and negative controls should be conducted for all samples (Hui 1993).

3.2.3. Immunological Methods

The interaction antigen and antibody has been preferred for many years to detect foodstuff adulterated and contaminated. Antigen-antibody reaction has specific role. This method is suitable for detection of chemical residues and antibiotic residues in animal foods. The enzyme-linked-immunosorbent assay (ELISA) is the most common method used. It is the well-established assay. Its detection system is usually depended on enzyme-labeled reagents. In ELISA test, color occurs during incubation. This color is measured with a micro plate reader (Toldra and Reig 2006). A low intensity indicates

positive result due to competitive principle. The result occurs less than 10 min (Neaves 1999).

Radioimmunoassay (RIA) performes the measure of radioactivity of immunological complex by using a counter. Other methods consist of chemiluminiscence measurement with a luminometer. In this duration, a chemiluminiscent compound is bound to the antibody or fluorescence with a fluorimeter (Toldra and Reig 2006).

Immunological test kits have advantages and disadvantages. Table 3.5 displays main advantages and disadvantages of these kits.

Table 3.5. Major advantages and disadvantages of ELISA test kits
(Source: Toldra and Reig 2006)

Advantages	Disadvantages
Easy to use	Increased costs since 2002 (more than
Available kits for a good number of specific	€6.50 per kit)
Compounds	Limited storage under refrigeration
Availability of kits for families of compunds	Expensive in the case of RIA and
Large number of samples per kit for a single	need for waste disposal
Analyte	Interferences giving some false
Reduced time to obtain the results	positives
High sensivity	Only one kit per residue searched
High specificity	
Possibility to use within the food-processing	
facility	

Another recent method is development of biosensors for screening antibiotic residues in dairy products. They have several constitutes. The target analyte contacts the biological receptor (antibody). The biochemical signal is converted by transducer into an electronic signal. A microprocessor displays the final result by using these signals. There are different types of biosensors. These are biochip array biosensors, enzymatic biosensors and biosensors based on antibiotic sensor protein that are convenient to specific classes of antibiotics. Biosensors have several advantages and disadvantages. The advantages are such as easy to use, to analyze multiples residues in short time for a large number of samples, full automatisation, computer controlled and high through-put

property. The disadvantages of biosensors are high initial equipment, high operative costs (chips) and analysis limited to available chips (Toldra and Reig 2006).

3.3. Chromatographic Methods

High performance thin layer chromatography (HPTLC) has been used for detection of multi-residues in food. However the usage of HPTLC has decreased. Different residues like thyreostatic drugs, clenbuterol and other agonists and sulfonamides have been determined by HPTLC in animal foods. Moreover the analysis of corticosteroids and antibiotics in milk has been conducted by means of HPLTC. Major advantages of HPTLC are as follows high number of samples for a single analyte, reduced time to obtain the results, possibility of automatisation for higher productivity, sensitive, specificity depending on the detection method, separated sample can be recorved for further confirmatory analysis. The drawbacks of HPTLC are that expertise required, need of sample preparation such as extraction and filtration, addition of internal standard, high initial equipment, cost of column (Toldra and Reig 2006).

The high performance liquid chromatography (HPLC) has been widely used for screening. HPLC is a separative method. It detects residues by means of detector. The detection system must be selected carefully. This system is significant for selectivity and sensitivity. The detection multi-residues are usually depended on a solid-phase extraction clean up and then filtration and injection into reverse-phase HPLC with UV-diode array detection. The main advantages and disadvantages of HPLC are shown in Table 3.6. (Toldra and Reig 2006).

Table 3.6. Main advantages and disadvantages of HPLC

(Source: Toldra and Reig 2006)

Advantages	Disadvantages
Short time to analyze	Expertise required
Sensitive	Need for sample preparation
Automatisation leading to higher	(extraction and filtration etc.)
productivity (injection, elution,	High initial equipment
washing of column, detection)	Cost of column
Possibility to find more information from	
spectra when using diode array detector	

HPLC is first screening method. The next step is injection of positive samples in a system combining HPLC with mass spectroscopy detection. HPLC with MS-MS can obtain the results in shorter time. The combination with HPLC-electro spray ionization (ESI) tandem mass spectrometry suggested as screening and confirmatory method. Some researchers have used liquid chromatography-mass spectrometry with atmospheric pressure chemical ionization (APCI) for the measurement. Another screening method for the analysis of antibiotics formulations is ^{1}H NMR (Toldra and Reig 2006).

Liquid chromatography and gas chromatography are other confirmatory methods. They coupled to mass spectrometry (LC/MS and GC/MS). They are highly specific and need complex equipment and well-qualified laboratory personnel (Okerman, et al. 2003). A clean-up is required before the chromatographic determination of antibiotics in milk. Antibiotics are typically polar constitutes and so are extracted into polar organic solvents. Precipitation of the milk proteins is required for the determination methods of antibiotics in milk (Schenk and Callery 1998). The principal drawback of the two methods is to use chemo-metrics analysis (Reid, et al. 2006).

CHAPTER 4

MILK CHEMISTRY AND PHYSICS

4.1. Introduction

Food safety issues and the potential for chemical and microbiological hazards in foods are paid attention by public. Consumers buy milk to be natural and wholesome. Severe financial difficulties on farmers are imposed by many countries. In some cases, veterinarians allow bulk tank milk to become contaminated with detectable levels of antibiotic residues (Hillerton, et al. 1999). Because financial losses result from decreased milk production, treatment, and labor costs, no deliverable milk, veterinary fees, reduced milk quality, reduced milk price, increased risk of subsequent mastitis, and increased risk of culling or death of the cow and also allergic reactions in human (Nielen, et al. 1992). For these reason, milk producers must preserve their milk. Milk must not be contaminated by any veterinary drugs prohibited or the levels of antibiotic residues were lower than the Maximum Residue Limits (MRLs) (Zvirdauskiene and Salomskiene 2007). And so, researchers investigate antibiotic free milk and milk spiked with antibiotic; and want to determine differences between them.

Milk is a complex chemical composition of water, lactose, fat, protein (mostly casein), minerals and vitamins distributed throughout colloidal and soluble phases (Fox and McSweeney 1998). Composition of cow's milk is so important for determining physical properties of milk because physical properties of milk change, according to composition. It is physical nature also is very complicated. It involves in three physical phases: a dilute emulsion, a colloidal dispersion, and a solution. If the emulsion is centrifuged, the milk separates into lipid and aqueous phases or compartments. These are each with a characteristic composition. If colloidal dispersion is centrifuged, casein micelles precipitate, bringing some other proteins, such as lactoferrin from milks of animal species (Neville and Jensen 1995).

The lactose, a portion of the mineral salts, and some of the lactalbumin are constituents of milk in molecular dispersion or true solution. Generally, the proteins of milk are dispersed colloidally through milk. Divided particles are casein, albumin and

globulin. Casein is the largest and albumin and globulin are the smallest. The calcium phosphate is considered with the proteins. It is colloidally dispersed. The fat of milk occurs in the form of a coarse dispersion or an emulsion. In milk, water is the continuous phase and because of this the emulsion is said to be of the fat-in-water type (Eckles, et al. 1951).

Table 4.1. Chemical composition of bovine milk

(Source: Otter 2003)

Component	Concentration (gl^{-1})
Lactose	36-55
Fat	
Triacyglcerols	36-38
Diacylglycerols	0.1-0.23
Monoacylglycerols	0.006-0.015
Sterols	0.09-0.16
Sterol esters	Trace
Unesterified fatty acids	0.04-0.17
Hydrocarbons	Trace
Phospholipids	0.08-0.39
Protein	30-35
Caseins	24-28
α_{s1}-Casein	12-15
α_{s2}-Casein	3-4
β-Casein	9-11
κ-Casein	2-4
Whey	5-7
β-Lactoglobulin	2-4
α-Lactalbumin	0.6-1.7
Bovine serum albumin	0.2-0.4
Immunoglobulins	0.5-1.8
Potassium	

Table 4.1. (cont.) Chemical composition of bovine milk

(Source: Otter 2003)

Casein fragments	
γ-Casein	1-2
Proteose-peptones	0.6-1.8
Milk fat globule membrane	0.4
Salt	0.7-0.8
Calcium	1.1-1.3
Chloride	0.9-1.1
Iron	0.3-0.6
Magnesium	0.09-0.14
Phosphorus	0.9-1.0
Sodium	0.35-0.9
Potassium	1.1-1.7

4.2. Physical Properties of Milk

Some of the physical properties of milk are summarized in Table 4.2 and explained in following sections.

Table 4.2. General physical properties of milk
(Source: National Dairy Council 2000)

Property	Value	Definition and Significance	Property	Value	Definition and Significance
Titratable acidity, % max	0.16	The total acidity or the amount of alkali required to neutralize the acidic constituents. Generally expressed as lactic acid. Used to determine bacterial growth in fermentations and compliance standards.	Specific heat at 0° C 15° C 40° C	0.92 0.94 0.93	The specific heat of milk products depends on their composition and the temperature. Important in processing as the amount of heat or refrigeration required may be calculated from the weight and specific heat of the different products being pasteurized or cooled.
pH	6.6 ± 0.2 at 25° C	Fresh milk is slightly acid (pH of drinking water is 7.0-8.5). Generally the pH is lower (pH 6.0) in colostrum and higher (up to 7.5) during mastitis than in normal milk of mid-lactation.	Coefficient of expansion at 10° C 15.6° C 21.1° C	0.9975 0.9985 1.0000	The ratio of an increase in volume per unit increase in temperature. Milk expands when heated and contracts when cooled. Used for design of dairy equipment.
Surface tension	50-52 dynes at 20° C	Normally, cow's milk's surface tension is about 70% of that of water. Involved in adsorption and formation and stability of emulsions. Important to creaming, functions of fat globule membranes, foaming, and emulsifier use.	Viscosity	2.0-2.1 cp at 20° C	Refers to resistance to flow measured in centipoise (cp). Used to assess aggregation of protein micelles or fat globules. Also used for design of dairy equipment.
Specific gravity	1.032 at 15° C	Ratio of the density of the product and the density of water at the same temperature. Many milk constituents have a specific gravity (sg) greater than that of water which has a sg of one. The more fat in milk, the lower the sg as fat has an sg less than one. Used to estimate solids not fat.	Electrical conductivity	$45\text{-}55 \times 10^{-4}$ mho	In milk, fat and colloidally dispersed substances decrease conductivity. Used to detect added neutralizers, follow fermentation, and monitor demineralization of whey.
Freezing point	-0.540° C	Lower than that of pure water (0° C) due to dissolved substances in milk. Used to detect adulteration of milk with water.	Osmolality*	275 m Osm/kg	The osmolality of a solution is based on the number of particles in solution – the greater the number of particles, the higher the osmolality. Osmolality of foods is important in planning diets of low osmolality for certain patients. Since a solution of lower osmolality requires transfer of less water to the stomach and gastrointestinal tract to dilute it, it should be better tolerated than one of higher osmolality.
Boiling point	100.17° C	Greater than that of pure water (100° C) due to dissolved substances in milk. Used to detect adulteration of milk with added water.			

4.2.1. Acid-Base Equilibrium

The acidity of solution is associated with the concentration of hydronium ions [H^+] in it. If the concentrations of [H^+] and [OH^-] (hydroxyl) ions are equal, the solution is called neutral. Mathematically, the pH can be defined as the negative logarithm of the hydronium ion [H^+] concentration.

Titratable acidity is pointed out the amount of alkali required to bring the pH to neutrality (phenolphthalein). This property is used in several fields. These are to determine bacterial growth during fermentations, such as during cheese making, as well as compliance with cleanliness standards. Fresh bovine milk has no lactic acid. Mostly, the titratable acidity is because of the casein and phosphates. Lactic acid can be produced by bacterial contamination in uncommonly (Neville and Jensen 1995).

Acidity, one of the most important parameters, controls the quality and processing of milk. Milk acts as a buffer. This buffer is a chemical system. It resists changes in the concentration of hydrogen ions under internal and external influences (Rosenthal 1991).

Most commonly, fresh milk and fermented products is tested analytically by measuring the amount of alkali needed to bring the pH to 8.4 (the end point of phenolphthalein which is used as indicator). The result is expressed by the equivalent amount of lactic acid. The solids-not-fat in the milk contains the more phosphates, proteins and other weak acids. pH value is largely a reflection of these. The more alkali is required to overcome the buffering capacity of milk over the range from the pH 6.6 up to 8.4. The titratable acidity of fresh normal milk should be about 0.14% lactic acid. If due to the microorganism' activity the milk is soured and the acidity is raised an extra amount of alkali is required. If the increase in titratable acidity is about 0.07% above the normal value, milk begins to taste sour. In a contrary manner, mastitis milk which may have an initial pH beyond 7, shows a titratable acidity of 0.1% lactic acid or less (Rosenthal 1991).

The titratable acidity of healthy cow milk milked newly 6.4-7.0 $^{\circ}$SH or 0.14%-0.16% in terms of milk acid (Metin 2001 and Rosenthal 1991).

The pH of milk as generally measured outside the animal because it is higher than milk within the mammary gland due to loss of CO_2 to the ambient air. The hydrogen-ion concentration of milk or dairy products may be determined different via

such as colorimetrically or electrometrically. There are numerous indicators available for the colorimetric method. Any one or combination of them is chosen depends upon the range of hydrogen-ion concentration to be measured. The electrometric method gives good results than the colorimetric method. The pH, actually, is the logarithm of the reciprocal of the hydrogen-ion concentration. On this scale neutrality is expressed by pH 7. If the figure is below 7 it indicates acidity and if above 7, alkalinity. It is not possible to convert titratable acidity directly into pH. There is some general relationship between the two measurements but each must be used and interpreted independently (Eckles, et al. 1951).

pH of bovine milk is 6,22-6,77 (Jensen 1995). Characteristic pH value at 25 $^\circ$C is 6.6, is reported range 6.5-6.7 (Webb, et al. 1974). The pH of healthy cow milk milked newly is 6.6 – 6.8. 0.2 is a small difference but it is very important in terms of hydrogen-ion activity. If the pH value of cow milk milked newly is above 6.8, to doubt is necessary due to mastitis or adding neutralized substance in milk. If pH value is smaller than 6.5, colostrum may exist or acidity may increase extremely and it means it is a problem for factory (Table 4.3), (Metin 2001).

Table 4.3. Degrees of acidity and properties of milk
(Source: Metin 2001)

Properties of Milk	$^\circ$SH	pH value
Milk with Mastitis	4.0 – 5.0	> 6.8
Normal Fresh Milk	6.5 – 7.5	6.6 – 6.8
Start of acidification	8.0 – 9.0	6.3
Coagulation by heating	10.0 – 12.0	5.7
Coagulated Milk	25.0 – 30.0	5.3 – 5.5

4.2.2. Specific Gravity

Milk is a complex colloidal system. The colloidal system involves in dispersion medium, water, contains salts and sugar in solution. Therefore, it is heavier than water (Eckles, et al. 1951). The density of milk is the weight of a unit volume at a defined temperature. Specific gravity is the density of a substance divided by the density of water at the same temperature. It is essentially 1.000 at 4 $^{\circ}$C. Water, nonfat solids and fat contents and also the degrees of fat crystallization or hydration of proteins are determined by using specific gravity. The density of milk decreases when the temperature is increased. Temperature controlling is important during the measurements. Water has a lower specific gravity than milk. When water is added to milk, the specific gravity decreases. However, a higher content of milk fat shows the same effect. Specific gravity is one of the most practical measurements used for controlling milk composition. Specific gravity of whole milk is 1.032, skim milk is 1.036, evaporated whole milk is 1.066 (Rosenthal 1991).

The specific gravity of milk usually is measured at 60 $^{\circ}$F (15.5 $^{\circ}$C) in the laboratory. Composition of milk affects the specific gravity of milk. Constituents of milk have different specific gravity, approximately: fat, 0.93; lactose, 1.666; proteins, 1.346; casein, 1.31; salts, 4.12 (Eckles, et al. 1951).

Density is so changeable because of all substances included in composition. The density of milk is 1.027 g/ml – 1.035 g/ml at 20 $^{\circ}$C due to its composition. The density of milk decreases because of increasing fat content and it increases because of decreasing fat content. The density of milk increases due to increasing quantity of protein, lactose and mineral substance. Increasing temperature causes decreasing density of milk (Metin 2001).

The specific gravity of milk is 1.021 – 1.037. The density of milk normally varies between 1.028 and 1.034 depending on composition. Milk is thus very slightly denser than water (Neville and Jensen 1995).

4.2.3. Electrical Conductivity

Electrical conductivity measurements have been used in the food industry for many years to detect contaminates in water, to monitor microbial growth, metabolic activity and to interference from inhibiting substances (Carcia-Golding and Giallorenzo 1995, Moreno and Chang 1995, Curda and Plockova 1995, Mitchell and Alwis 1989). In addition, electrical conductivity measurement is widely used in dairy industry. For instance, detection of mastitis for quality control of milk, to analyze fermentation processes for production of cheese starters (Paquet, et al. 2000), to monitor the start-up and prerinsing phases of milk pasteurization process (Henningsson, et al. 2005)

Electrical conductivity is a measure of the resistance of a particular material to an electric current. The conductivity is the reciprocal of resistance. Resistance is measured in ohms and is calculated by dividing voltage by amperes. Conductivity is measured in Siemens and is calculated by dividing ampere by voltage (Nielen, et al. 1992).

Milk has conductive properties due to the existence of charged compounds, especially mineral salts. The distribution of salt fractions between the soluble and colloidal phases has an important effect on milk conductivity value. The electrical conductivity of milk is determined primarily by not only sodium and chloride ions but also by other ions (Mucchetti, et al. 1994, Mabrook, et al. 2006). There is very little associated with the lactose as the conductance values of full fat milk. The salts in milk contain mainly of chlorides, phosphates, citrates, carbonates and bicarbonates of potassium, sodium, calcium, and magnesium, although the salt content of milk remains constant at about 0.7% w/v. This composition is affected by factors such as animal breed, season of the year, feed, and stage of lactation (Fox and McSweeney 1998). These factors also influence the distribution of calcium, magnesium, and phosphate between soluble and colloidal phases and thus the number of free conducting ions in the milk. Despite casein, the main milk protein shows a very low conductance compared to the milk salts. The insoluble salts in milk, especially calcium phosphate, are mainly correlated with the casein micelles in the colloidal phase. Small rates of the sodium and potassium ions are associated with the casein as counter-ions to the negatively charged organic phosphate groups of the protein (Fox and McSweeney 1998). These salts act like bridges between the subunits of the casein micelles. They keep the milk in a stable

condition. Under certain conditions, these salts can be released into solution in that connection increasing the conductivity (Mabrook and Petty 2003).

Moreover, when acidity increases, conductance of milk increases. In an experiment, freshly squeezed lemon juice was added slowly to full fat milk and at the some time conductance and pH were monitored. The conductance increased, reaching a saturation value of 5.8 mS at a pH of 4.9-5.0. This conductance value is similar to milk gone off. At this situation, all the colloidal salts connected to the casein micelles are in a soluble phase and free to contribute to the measured conductance. The addition of acid decreases the pH of the milk. It results solubilisation of the colloidal salts connected to the casein micelles gradually. When the pH of milk reaches a value of about 5.0, all the colloidal calcium and phosphorus are in the soluble phase and the conductance saturates (Mabrook and Petty 2003).

The presence of fat is another factor that has an influence on the electrical conductance of milk (Lawton and Pethig 1993, Prentice 1962). The conductance of milk decreases when the percentage of fat increases. Conductance of full fat milk [3.6 wt%] , semi-skimmed milk [1.6 wt%], skimmed milk [0.1 wt%] is 5.05 +/- 0.03 mS, 5.23 +/- 0.03 mS, 5.4 +/- 0.03 mS , respectively (Mabrook and Petty 2003).

Electrical conductivity of normal milk, with a temperature of 25 $^\circ$C, is typically between 4.0 and 5.0 mS/cm (Wong 1988). When measured in-line, EC will have some higher level on account of the higher temperature in the milk (38°C). Numerous studies show that EC of milk from cows affected by mastitis (both clinically and subclinically). This EC of milk is higher than EC of milk from healthy cows (not affected by mastitis) (Nielen, et al. 1992, Hamann and Zecconi 1998, Norberg, et al. 2004a, Biggadike, et al. 2000). The mean EC in mS (with standard errors in parentheses) for healty, subclinically infected and clinically infected cows was 5.3 (+/-0.03), 5.75 (+/-0.04) and 6.73 (+/- 0.06), respectively. These numbers were significantly different (P<0.001) (Norberg, et al. 2004a). Electrical conductivities of raw milk and skim mil k are stated 4680, 4920 μ S/cm, respectively (Mucchetti, et al. 1994).

Typical EC of normal milk appears to be between 4.0 and 5.5 mS/cm at 25 $^\circ$C, and the distribution of the measurements is log normal. The EC of milk has also been expressed as a concentration of NaCl because of the same conductivity. Milk was examined in milli moles. It reflects the total ionic concentration of the milk in milliequivalents per liter (Nielen, et al. 1992). Table 4.4 indicates about the electrical conductivity values of milk, each of the components and fractions of milk.

Table 4.4. EC of milk, milk fractions and constitutes

(Source: Mucchetti, et al. 1994)

Sample	
	(μS/cm)
Raw milk	4680
Skim milk	4920
Whey	5180
Diafiltered milk	278
Permeate UF	4762
Whey protein solution, 78%	214
Lactose solution, 5%	27
AA Solution, 6.25 mM	
Glutamic acid	100
Arginine	124
Cysteine	8
Methionine	7
Urea solution, 50 mg/100 ml	4
Salt solution, 100 mg/100 ml	
NaCl	1626
KCl	1585
Na lactate	516
$CaCl_2$	1475
Na_3 Citrate	869
K_2HPO_4	1270

4.3. Thermo-Physical Properties of Milk

The thermal analysis has been preferred for many years to characterize synthetic polymers. However, there is a growing interest to use thermal analysis techniques to characterize food systems. In food science, thermal analysis has already been used for detection of water loss, uptake or migration, the denaturation of proteins and the crystallization of starch, to design new processes like freeze-drying, spray-drying, extrusion and hydro-thermal treatment and also for determination and improvement of food quality, safety and storage stability. Differential scanning calorimetry (DSC) is one of the most popular thermal analysis techniques in which the difference in the amount of heat required to increase the temperature of a sample and reference are measured as a function of temperature. Both the sample and reference are maintained at nearly the same temperature throughout the experiment. However, there are some problems regarding interpretation of DSC curves. These problems in most foods are the low purity, high heterogeneity, polydispersity, water content and weak, broad transitions (De Meuter, et al. 1999). The significant factors affecting thermal properties are generally food composition and temperature (Tansakul and Chaisawang 2006). The specific heat of foods depends strongly on the water content. Water has the highest specific heat of all food components. Generally, specific heat measurements are made by using calorimeter. It is a simple technique but it requires a correct calibration. The best alternative technique is differential scanning calorimeter. DSC determines the specific heat of foods experimentally. The disadvantage of DSC is being expensive (Telis-Romero, et al. 1998).

4.3.1. Specific Heat

All substances have different chemical and physical nature. For these reasons, the amount of heat required varies. The specific heat is the ratio between the amount of heat necessary to raise a given weight of a substance to a specified temperature and the amount of heat required to raise an equal amount of water to the same temperature. Calorie is the unit of heat (Eckles, et al. 1951). The specific gravity of water is the greatest at 3.9 °C. At this temperature, the specific heat of water is taken as the standard. Other substances can be compared by using this (Eckles, et al. 1951).

The specific heat of milk is 0.938 kJ/kg K at 15 °C, 0.920 kJ/kg K at 0 °C, 0.930 kJ/kg K at 40 °C and 0.918 kJ/kg K at 60 °C. The specific heat of milk products is various from each other because of their chemical composition. The heat capacity of whole milk and cream with temperature is more complicated than skim milk due to the milk fat effect (Hui 1993). It is significant in processing for determining the amount of heat and cooling required changing the temperature of milk (Jensen 1995). Moreover, engineers need the specific heat to calculate costs in the heating and cooling of milk and milk products (Eckles, et al. 1951).

4.4. Chemical Properties of Milk

The principal compounds of milk are water, fat, proteins, lactose (milk sugar) and minerals (salts). In addition, milk includes trace amounts of other substances such as enzymes, pigments, vitamins, phospholipids (substances with fat like properties), and gases. When gases and water are removed is called the dry matter or total solids content of the milk, the residue left (Table 4.5) (Eckles, et al. 1951).

Table 4.5. Chemical composition of milk

(Source: Eckles, et al. 1951)

		Percentage
Water		87.25
Dry matter		12.75
Fat	3.80	
Protein	3.50	
Sugar	4.80	
Ash	0.65	
Total		100.00

4.4.1. Milk Fat

Milk fat is the most valuable component of milk. Milk and milk products have rich flavor because of the milk fat. Milk fat observes in milk in the form of minute globules in a true emulsion of the oil-in-water type. The fat globules exist in the dispersed phase. If the milk is examined under a high- power microscope, the numbers of fat globules in various sizes will be seen. The breed is the most important factor that affects the size of the globules. The fat globules are almost large during the first phase of the lactation period. The size of the fat globule is of significance for separation of milk, churning of cream, shipping of milk or cream and cheese making (Eckles, et al. 1951).

Milk fat has complex chemical composition. It is a variable mixture of several different glycerides. The glycerides contain the union of glycerol and one or more organic acids. Milk fat involves a larger number of the fatty acids. These are butyric, caproic, caprylic, capric, lauric, myristic, palmitic, stearic and oleic acids. The oleic acid is the greatest amount of total fatty acids in milk (Eckles, et al. 1951). The breed of animal, stage of lactation, feed and plane of nutrition are responsible for this composition of milk fat.

Milk fat is a triglyceride accounted for 98.3% (Hui 1993) that contains 1 molecule of glycerol combined with 3 molecules of the fatty acid. The fatty acids of milk divide into two general groups. These are the volatile and the non-volatile. The volatile acids involve butyric, caproic, caprylic, capric, lauric, and small amounts of others. The non-volatiles are myristic, palmitic, oleic, stearic and small amounts of a few others (Eckles, et al. 1951).

All of the fatty acids in milk fat have different melting points, boiling points and specific gravity. In other words, the melting point, boiling points and specific gravity of milk fat is influenced by its composition (Eckles, et al. 1951).

The physical properties of milk fat have been stated such as: density at 20 °C is 915 kgm^{-3}; refractive index (589 nm) is 1.462 and decreases with increasing temperature; solubility of water in fat is 0.14% (w/w) at 20 °C and increases with increasing temperature; thermal conductivity is about 0.17 $J\,m^{-1}s^{-1}K^{-1}$ at 20 °C; specific heat at 40 °C is about 2.1 $kJ\,kg^{-1}K^{-1}$; electrical conductivity is $<10^{-12}$ $ohm^{-1}\,cm^{-1}$; and the dielectric constant is about 3.1 (Hui 1993).

4.4.2. Proteins of Milk

Proteins are one of the complex organic substances (Eckles, et al. 1951). Proteins consist of amino acids. Carbon, hydrogen, oxygen and nitrogen are the elements of amino acids (Rosenthal 1991). Nitrogen distribution of milk is divided among caseins, whey proteins and non-protein (NPN) (Hui 1993). The protein content of milk is approximately 3% (Eckles, et al. 1951).

Casein is a yellowish-white granular substance. Pure casein is snow-white, odorless and tasteless. It is observed in combination with calcium in milk. Casein is found in colloidal form (Eckles, et al. 1951). The casein content of milk is about 80% of milk protein (Hui 1993). Casein is widely used in industry including manufacturing of plastics, sizing of high-grade paper, textile industry and food industry such as meat sauce, baby foods (Eckles, et al. 1951).

The whey proteins fractions are β-lactoglobulins, α-lactalbumins, bovine serum albumin and immunoglobulins (Hui 1993). Lactalbumin is one of the important fractions of whey. Lactalbumin dried is a tasteless powder. It consists of carbon, oxygen, hydrogen, nitrogen and a small quantity of sulfur (Eckles, et al. 1951). α-Lactalbumin has a vital role in milk composition. β-lactoglobulin has been known as the allergenicity of milk (Rosenthal 1991).

Milk proteins are essentially rich in essential amino acids. These are phenylalanine, methionine, leucine, valine, lysine, isoleucine, threonine, tryptophan, histidine. Lysine is one of the abundant essential amino acids in milk proteins (Rosenthal 1991).

4.4.3. Lactose

Lactose is a milk sugar. It is a carbohydrate and one of the most common disaccharides. It contains 1 molecule of galactose and 1 molecule of glucose. Lactose is in the milk serum. Milk consists of about 4.8 percent of lactose. Dry milk involves a great amount of percentage of lactose, about 38% (Eckles, et al. 1951).

Many other sugars are more soluble than lactose. Sucrose is sweeter than lactose. The sweetness of α-lactose is not greater than that of β-lactose. Lactic acid is

the metabolite of lactose. It is produced microbiologically in milk and it is the main source of energy for microbial metabolism (Rosenthal 1991).

4.4.4. Minor Components of Milk

In addition to the major components, protein, lactose, water, fat and ash, milk involves some minor components of milk (Eckles, et al. 1951). Minor components of milk are some vitamins, minerals, nonprotein nitrogenous substance, phosphoric esters, ethanol, cholesterol, pigments, enzymes, gases and some acids (Hui 1993).

4.4.4.1. Vitamins

Milk contains both fat and water-soluble vitamins. Milk consists of vitamin A, vitamin B_2, vitamin B_1 (thiamin), vitamin G (riboflavin), nicotinic acid (niacin), vitamin B_6 (pyridoxine), pantothenic acid, vitamin C (ascorbic acid), vitamin D, vitamin E (alpha-tocopherol) and vitamin K. The feed affect the amount of some vitamins in milk (Eckles, et al. 1951).

4.4.4.2. Minerals

22 minerals are considered to be essential to the human diet are present in milk. Three families of salts are included in milk. The first one includes sodium (Na), potassium (K) and chloride (Cl). A second includes colloidal calcium (Ca), magnesium (Mg), inorganic phosphorus (P_i) and citrate. The third family includes diffusible salts of Ca, Mg, citrate and phosphate (Hui 1993).

4.4.4.3. Enzymes

The enzyme in milk can occur from the mammary gland as native component or may be resulted by contaminating bacteria. Milk involves lipase, alkaline phosphatese, lactoperoxidase and catalase. Lipases catalyze the hydrolysis of milk fat to free fatty acids and glycerol. Alkaline phosphatese catalyzes the cleavage of phosphoric acid ester into phosphoric acid and hydroxylic constituents, alcohols or phenols. Lactoperoxidase

catalyses the transfer of oxygen from peroxides, particularly hydrogen peroxide, to other oxidizable substances. Catalase affects the decomposition of hydrogen peroxide to water and molecular oxygen (Rosenthal 1991).

4.4.4.4. Non-protein Nitrogenous Substances

In addition to the milk protein, milk consists of nitrogenous substances. These substances are the rest in small size compounds. These are urea (2.5%), uric acid (0.5%), amino acids (0.8%), amines, ammonia, creatinine, adenine and guanine. These constitutes do not have biological value as proteins (Rosenthal 1991). These non-protein nitrogenous substances are measured in parts per million (1.5 to 10) in milk (Eckles, et al. 1951).

CHAPTER 5

MATERIALS AND METHODS

5.1. Materials

According to unofficial data, most of the antibiotic containing raw milk is processed to UHT milk in Turkey. This may be due to the fact that at high temperature, antibiotics are degraded and their level reduced under detection limits. That is why UHT milk having 3.1% fat content was selected for analyzes.

UHT whole cow's milks were obtained from the local market in Izmir, Turkey. The production date, expiry date, serial number, energy and nutrition values of milk were recorded. Milk samples were supplied between 2006 and 2008. The milk samples were kept refrigerated before analysis. Prior to measurements, the samples were kept at room temperature.

To investigate physical and chemical properties of whole antibiotic free milk and whole milk spiked with antibiotics, Penicilline G potassium salt (46609, Lot 4016X), Ampicillin trihydrate (46061, Lot 2316X) and Tetracycline hydrochloride (46935, Lot 3301X) (Vetranal analytical standard Sigma-Aldrich GmbH Quality Assurance) were chosen as target antibiotics in this study. Penicillin G and Ampicillin were prepared in the concentration of 2, 4, 8 ppb and whereas tetracycline was prepared in the concentration of 100, 250 and 500 ppb.

The CMT Copan Milk Test (Copan, Italy), Penyzme Test Kit (UCB-Bioproducts, Belgium) and ROSA Test Kit (Charm Science Inc., USA) were used to screen milk samples for antibiotic residues using procedures recommended by the manufacturer. HPLC, LC-MS and LC-MS/MS were used for confirmatory analysis of milk samples carried out in Bornova Veterinary Control and Research Institute, Izmir, Turkey.

5.2. Methods

5.2.1. CMT Copan Milk Test Procedure

This assay is based on the rapid growth and acid production of test organism, *Bacillus stearothermophilus* var. *calidolactis* C 953 spores.The CMT Copan Milk Test is supplied in individual tubes and multi-well micro plates filled with an agar medium. The agar is pre-seeded with spores of *Bacillus stearothermophilus* var. *calidolactis* and incorporates a fermentable sugar, glucose and a pH indicator, Bromocresol Purple. The test is ready to use with no necessity to activate the product by adding a nutrient tablet. Detection limits and MRL's for target antibiotics are presented in Table 5.1.

Table 5.1. Detection limits and MRL's for Copan Milk Test
(Source: Copan 2008)

Antibiotics	Copan Test Detection Limit (ppb)	MRL (ppb)
Penicillin G	1 - 2	4
Ampicillin	< 2	4
Tetracycline	250 - 500	100

100 μ l of milk to be tested was added directly onto the surface of the agar and then incubated at 64 ± 0.5 $^{\circ}$C in an incubator for a prescribed length of time. After the incubation time of 3 hrs ± 15' the results were read.

The milk quickly diffuses throughout the agar medium. If there are no antimicrobial substances in the milk sample or the concentration is lower then the limits of detection the *Bacillus* spores germinate, grow and metabolize the sugar. The acid produced from the fermentation of glucose changes the color of the indicator Bromocresol Purple in the medium to a yellow color. Alternatively, if antimicrobial substances are present in the milk sample then germination and growth of the Bacillus spores is inhibited. This means there is no fermentation of glucose, acid production and

therefore the Bromocresol Purple indicator in the medium remains a purple color. Yellow/Purple (Partially Positive) means that no inhibitors are present or that the presence is lower than the limit detectable by the test (Figure 5.1.).
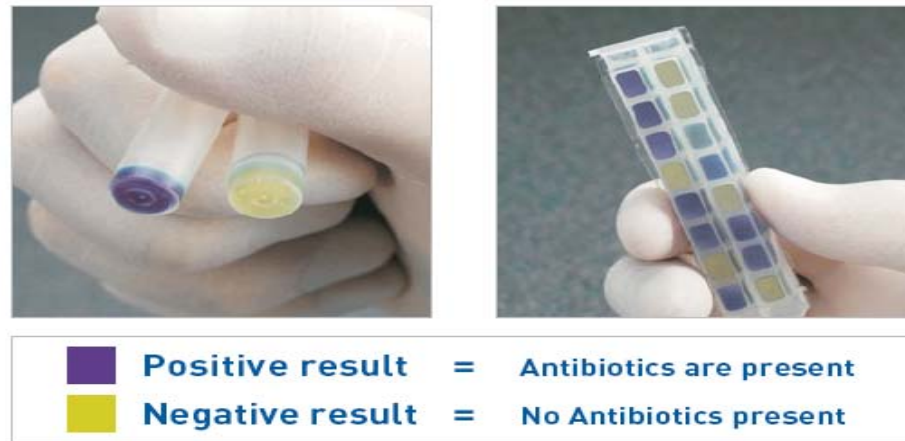
Figure 5.1. Copan milk test results

(Source: Copan 2008)

5.2.2. Penzyme Milk Test Kit Procedure

Penzyme is an enzyme colorimetric assay for rapid determination of beta-lactam antibiotics. The test consists of an enzyme DD-carboxypeptidase. DD-carboxypeptidase hydrolyzes synthetic substrate of type R-D-Ala-D-Ala and rapidly reacts with beta-lactam antibiotics to form a stable, inactive complex. Five-minute incubation of a specific amount of enzyme with milk sample involving antibiotic results in the inactivation of a certain amount of the enzyme. Due to the inactivation of the enzyme, no reaction occurs when the vial incubates for second reading. The vial is observed yellow color. Yellow color indicates the presence of beta-lactam antibiotics. If no antibiotic is present, the enzyme remains active form and reacts with the substrate. Pink or orange color occurs (Seymour, et al. 1988).

10 µl of enzyme (DD-carboxypeptidase in its buffer) is added into 50 µl of milk and then is mixed and incubated for 5 minutes at 47 °C. Secondly, one tablet with all the other reagents needed in the assay is added and flick slowly the tube to bring the tablet to the bottom. It is incubated at 47 °C again. The first reading is made after 8 minutes incubation. If the tablet shows a color between pink orange and the peach, the sample is

considered as negative. On the other hand, if yellow color between the peach or yellow color is obtained, the sample probably contains antibiotics. After the first reading, the sample is placed in incubator for 7 additional minutes. The color of samples is read by comparing it to the color chart. When the color is pink orange, it means the sample is negative. This color is characteristic of samples with Penicillin G concentrations lower than 0.005 IU per ml of milk. When the color of the sample is peach, the concentration is about 0.008 IU Penicillin G per ml of milk. Such a sample is caution sample. When the color of the sample is yellow, this sample is considered as positive. This means the samples contains Penicillin G concentrations higher than 0.017 IU per ml of milk. Table 5.2 displays an interpretation of the colors at the second reading.

Table 5.2. The colors at the seconding reading

Colour	Result
Pink orange	Negative
Peach	Caution
Between peach and yellow	Positive
Yellow	Positive

5.2.3. ROSA (Rapid One Step Assay): MRL Test Procedure

The Charm MRL test is a rapid assay conducting ROSA (Rapid One Step Assay) technology. This test is developed to detect beta-lactam and tetracycline antibiotics at the EU/Codex maximum residue limits (MRLs) in milk (Charm 2008). Table 5.3 indicates detection levels in cow's milk.

Table 5.3. Detection levels in cow's milk

(Source: Charm 2008)

Antibiotics	Detection Level Range (ppb)	EU / Codex MRL (ppb)
Penicillin G	2 - 3	4
Ampicillin	3 - 4	4
Tetracycline	30 - 90	100

Principle of ROSA as follows: 300 µl of milk sample is added to the sample well. The milk is absorbed by the orange sponge and transferred to the porous paper. As the test is incubated the milk travels up the paper and picks up the purple indicator beads which are located just above the orange sponge. These beads are going to bind either to the test line (will develop next to the T) or the control line (located further up the strip next to the C), (Figure 5.2). If T line is same as or darker than C line, this means negative sample. If T line is clearly lighter than C line, or T line is absent, or partially or unevenly colored, this result indicates positive sample (Figure 5.3.), (Charm 2008).
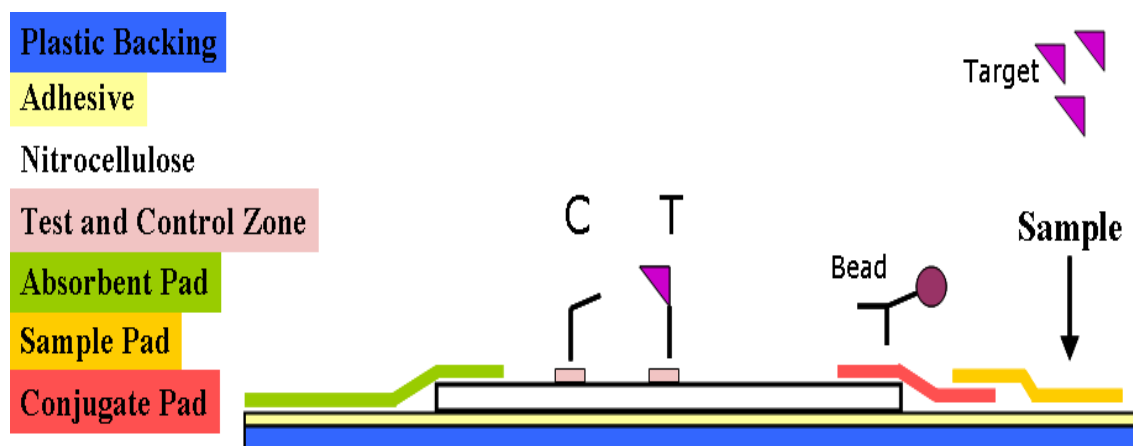
Figure 5.2. ROSA test design

(Source: Charm 2008)
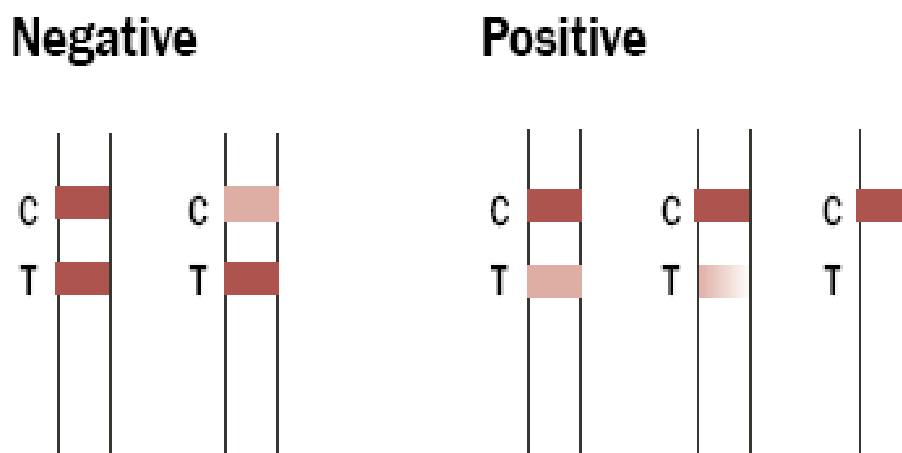
Figure 5.3. Visual interpretation of ROSA test

(Source: Charm 2008)

5.2.4. Preparation of Antibiotic Standard Solution

Penicillin G potassium salt ($C_{16}H_{17}KN_2O_4S$, 99.4%, 372.48 g/mol), Ampicillin trihydrate ($C_{16}H_{19}N_3O_4S.3H_2O$, 98.1%, 403.45 g/mol) and Tetracycline hydrochloride ($C_{22}H_{25}ClN_2O_8$, 97.3%, 480.90 g/mol) were used to prepare standard solution. Working standard solutions of penicillin G (2, 4, 8 ppb), ampicillin (2, 4, 8 ppb) and tetracycline (100, 250, 500 ppb) concentrations were prepared by diluting the stock standard solution with water (Sivakesava and Irudayaraj 2002). The reason of selecting such levels of antibiotic concentrations was that 'one was under MRL level, one was at MRL and one was above MRL limits'.

5.2.5. The Acidity Test

The acidity of milk is determined by titration with a tenth-normal solution of an alkali, such as sodium hydroxide. Each milliliter of a tenth-normal solution of sodium hydroxide neutralizes 1 milliliter of a tenth-normal solution of lactic acid. The percentage of lactic acid of milk is calculated. 1 milliliter of a tenth-normal lactic acid contains 0.009 gram of lactic acid. In other words, 1 ml N/10 NaOH neutralizes 0.009 gram of lactic acid. It is multiplied number of milliliters of a tenth-normal alkali necessary to neutralize the lactic acid in the sample. It gives the number of grams of

lactic acid in the milk sample. When this result is divided by the total number of grams of milk in the sample and multiplied by 100, the percentage of lactic acid in the sample will be obtained. This calculation may be illustrated more clearly by the following formula (Eckles, et al. 1951):

$$\% \text{ of lactic acid} = \frac{millilitersN/10x0,009}{gramsofsample} \qquad (5.1)$$
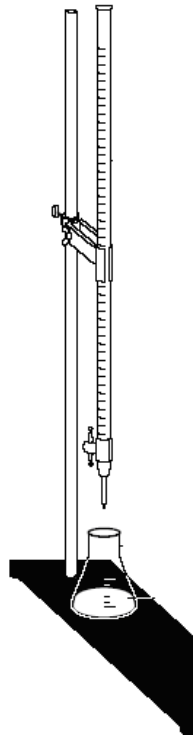
Figure 5.4. Titration mechanism

18 gr milk is weighed into the erlenmeyer flask, 0.5 milliliter of the phenolphthalein solution (1%, 1 percent alcoholic solution of phenolphthalein) is added as an indicator. N/10 of NaOH solution is added next. The reading of NaOH in the burette at the lowest point of the meniscus is noted, at the same time, the contents of the erlenmeyer flask are stirred constantly (Figure 5.4). When a faint but definite and persistent pink color appears, this indicates the end point (Kırdar 2001). The percentage of lactic acid in the sample was calculated from the formula given in 5.1.

5.2.6. Determination of pH

The hydrogen-ion concentration of milk can be determined calorimetrically or electrometrically. In the electrometric method, a pH-meter is widely used. The electrometric method gives greater precision than the colorimetric method (Eckles, et al. 1951).

For measurements, pH was measured by a Mettler Toledo SevenEasy pH-meter (Mettler Toledo, USA). The reference temperature is accepted 25 ^{o}C. All measurements were performed two times.

5.2.7. Determination of Density

The density of milk and milk products is used for converting volume into mass and vice versa, estimating the solids content and calculating other physical properties (e.g. kinematic viscosity). Density is depended on temperature at the time of measurement, temperature history of the material, composition of the material (especially the fat content) and inclusion of air (Guelph 2005).

All is considered, density of milk samples was measured by DA-130N KEM Density / Specific Gravity Meter (Kyoto Electronics, Japan). All measurements were performed in three replicates. The densities of milk samples were carried out at 15, 20, 25, 30, 35, 40, 45, 50 $^{°}$C and were observed changes based on temperatures. Different temperatures were regulated by water bath.

5.2.8. Determination of Electrical Conductivity

Electrical conductivity was measured by EC215 Bench Conductivity Meters conductometer (Hanna Instruments, USA). EC meter was calibrated using HI 7031 Conductivity Calibration Solution (1413 μS/cm 25 $^{°}$C / 77 $^{°}$F, Hanna Instrument, Hungary). Antibiotic free milk and milk containing Penicillin G (2, 4, 8 ppb), Ampicillin (2, 4, 8 ppb), Tetracycline (100, 250, 500 ppb) were prepared at sterile medium. The milk samples were poured into 250 mL plastic flask. All measurements were performed in five times. The reference temperature was accepted 25 $^{°}$C (Zhuang, et al. 1997).

5.2.9. DSC Analysis

Q10 DSC (TA Instruments, USA) was used to determine the thermal behavior of milk samples. Prepared milk samples consist of Penicillin G (0, 2, 4, 8 ppb), Ampicillin (0, 2, 4, 8 ppb), Tetracycline (0, 100, 250, 500 ppb). DSC was previously calibrated using standard. The heating and cooling were made under a constant nitrogen flow. The experimental conditions (temperature range, type of crucible, temperature programming, heating rate (10 °C min^{-1}), cooling rate (10 °C min^{-1}), and weighed mass of sample) was described in DSC software program (Cordella, et al. 2003). Approximately 5-10 mg of the samples were weighed and hermetically sealed into an aluminum pan by using a sealer. Then, DSC runs were performed to determine thermal parameters of the samples. The samples were cooled with liquid nitrogen as a cooling medium and scanned from – 30 °C to 250 °C (De Meuter, et al. 1999).
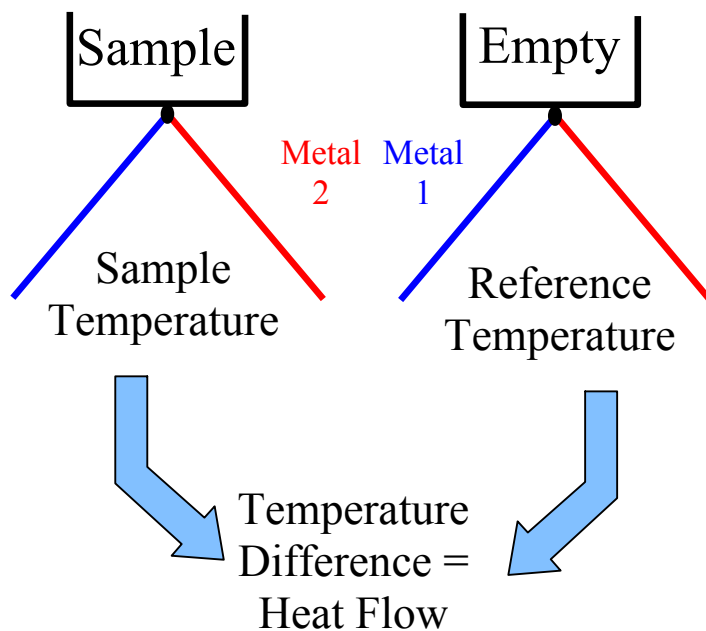
Figure 5.5. DSC mechanism

(Source: Collins 2008)

The basic principle of this technique involves the sample to undergo a physical transformation such as phase transitions, more or less heat will require to flow to it than the reference to maintain both at the same temperature. The temperature program for a

DSC analysis was designed that the sample holder temperature increases linearly as a function of time (Figure 5.5).

5.2.10. Yogurt Culture Test

The milk samples were inoculated with a yogurt culture into NU-425-400E, Class II biological safety cabinets (Nuaire, Inc., USA). Yogurt cultures involved equal mixtures (2%) of *Streptococcus thermophilus* (St 95/1) and *Lactobacillus delbrueckii* sup-sp. *bulgaricus* (Lb 54). They were isolated from traditional yogurt samples of Toros mountain region of Turkey. Phenotypic and genotypic characterizations of the cultures were performed by Molecular Food Biotechnology research group at Izmir Institute of Technology (Erkus, et al. 2006). Milk samples were incubated at 43 °C for 6 h. Firstly, pHs of all milk samples were determined at 0. hour. Then, antibiotic free milk samples and milk containing Penicillin G (2, 4, 8 ppb), Ampicillin (2, 4, 8 ppb), Tetracycline (100, 250, 500 ppb) were incubated for 6 hours. During the incubation, duplicate measurements of pH were made immediately at 2., 4., 6. hour using Mettler Toledo SevenEasy pH meter (Mettler Toledo, USA). The decrease of milk pH was observed and compared with each other (Yamani, et al. 1999).

5.2.11. Determination of Fat, Protein, Lactose, Minerals, SNF (Fat-Free Dry Matter) and Freezing Point

The percent of protein, lactose, minerals, SNF and freezing point of milk samples are determined by using Funke Gerber 3510 Laktostar milk content analyzer (Funke Gerber, Berlin, Germany). Lactostar has been newly developed for the routine testing of milk. It makes fully automatic cleaning, flushing and fully automatic zero-point calibration. It consists of four measurement cells. These measurement cells are divided in two measurement units. The measurement is depended on a thermo-optical procedure combination. The milk sample (12 to 20 ml) is pumped in two different measuring cells. It is anayzed by means of these two measuring units. These are blue box (opto-unit) and red box (thermal unit). This indicates that the milk samples are analyzed by using to completely different measuring method. The blue box is a turbidity measurement. In this measuring unit, the undissolved (visible) substances are analyzed.

For instance, it measures the sum of fat and protein. It involves in impedance or conductance measurement. Red box contains two thermo analytical measurement cells. In this cell, measurement occurs two different measurement temperatures (40.00 °C / 65.00 °C). The fat content and the fat-free dry matter are measured through thermal effects at different measuring temperatures (Figure 5.6).
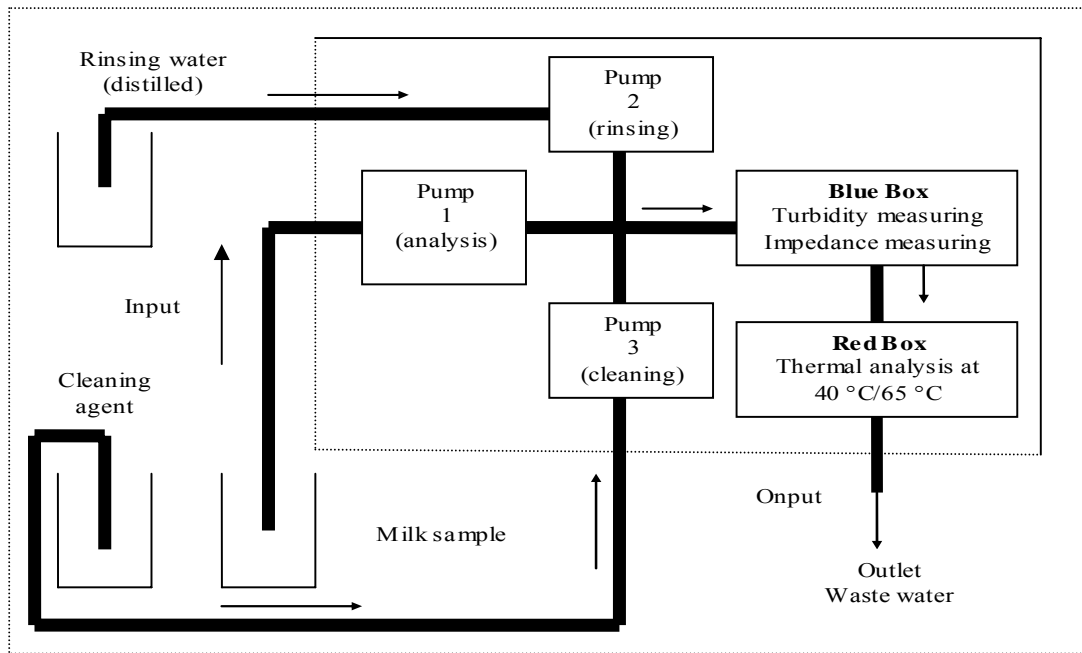
Figure 5.6. Lactostar mechanism
(Source: Bentleyczech 2008)

The content of antibiotic free milk samples and milk containing Penicillin G (2, 4, 8 ppb), Ampicillin (2, 4, 8 ppb), Tetracycline (100, 250, 500 ppb) was determined at 60 sec tempering time and 15 sec measurement time. All determinations were performed five times.

5.2.12. Confirmatory Methods

According to The International Union of Pure and Applied Chemistry (IUPAC), chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary phase, while the mobile phase moves in a definite direction. Mobile phase means as "a fluid that percolates through or along the stationary bed in a definite direction" such as liquid,

gas. The stationary phase may be a solid, a gel or a liquid. Chromatographic system mainly consists of a device for sample introduction, a mobile phase, a stationary phase and a detector.

There are two significant terms, the selectivity of a detector, and the limit of detection. The selectivity of a detector is its ability to determine an analyte of interest without interference from other materials present in the analytical system, i.e. the sample matrix, solvent used. The limit of detection is the smallest amount of an analyte that is required for reliable determination, identification or quantization. Figure 5.7 shows a block diagram of an HPLC system with its major components (Ardrey 2003).
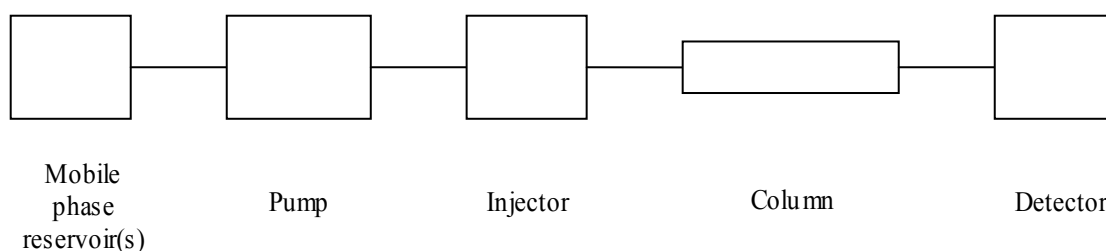
Figure 5.7. A typical HPLC system

(Source: Ardrey 2003)

5.2.12.1. Liquid Chromatography Detection of Penicillin G in Milk

5.2.12.1.1. Reagents, Standards and Apparatus for LC Analysis

Penicillin G potassium salt (99.4%) was obtained from Riedel-de-Haën (Sigma Aldrich GmbH Quality Assurance, Germany). Methanol, acetonitrile and isooctane were of analytical grade (Merck, Germany). Ultra pure water for LC analysis was obtained with an ultrafiltration unit system (ELGA Labwater, UK). Disodium hydrogen phosphate dihydrate, dibasic sodium phosphate dehydrate, sodium thiosulfate pentahydrate, hydrogenosulfate tetrabutylammonium and mercuric chloride were purchased from Merck. 1,2,4-triazole was purchased from Sigma.

A phosphate buffer (pH 6.5, 0.1 mol l^{-1}) containing 0.015 mol l^{-1} thiosulfate and 0.02 mol l^{-1} tetrabutylammonium hydrogenosulfate was prepared by mixing and dissolving 6.229 g disodium hydrogen phosphate dehydrate, 10.139 g dibasic sodium

phosphate dehydrate, 3.894 g sodium thiosulfate pentahydrate and 6.791 g tetrabutylammonium hydrogenosulfate in 800 ml water. This solution was diluted to volume in a 1 l volumetric flask, mixed thoroughly and filtered under vacuum through a 0.45 μm unit (Sartorius Minisart RC 15, Goettingen, Germany). It was stored at +4 °C and used for no more than 5 days.

When mixed by the pump, the mobile phase contained 650 ml of the 0.1 mol/l phosphate buffer pH 6.5 and 350 ml acetonitrile.

A phosphate buffer extraction solution (pH 8, 0.1 mol l^{-1}) was prepared by dissolving 14.196 g disodium dibasic sodium phosphate dehydrate in 1 l ultra-pure water.

The elution solution (50:50) for the solid-phase extraction (SPE) step was prepared each day by mixing 50 ml of water with 50 ml acetonitrile.

The derivatizing reagent (2 mol/l 1,2,4-triazole and 0.01 mol/l mercuric chloride) was obtained by weighing 13.78 g 1,2,4-triazole in a 100 ml beaker, adding 60 ml water, stirring to dissolve. This solution was mixed with 10 ml 0.1 mol/l mercuric chloride solution, adjusted to pH 9.0 ± 0.1 with 5 mol/l NaOH, transferred to a 100 ml volumetric flask, and diluted to volume with water. When refrigerated at 4 °C and protected from sunlight, the derivatizing reagent can be stored for up 2 months.

Stock solution of Penicillin G was prepared by dissolving pure reference standard in water. Working standard solution was stored in cool place protected from light. Stock solutions and working standard solutions were prepared fresh every 2 weeks to prevent degradation. A calibration curve was constructed using standard solution by diluting 100 µl of the working standard solution with 900 µl of the SPE (50:50) into 5 ml glass tubes to obtain 4 levels of Penicillin G concentration of 40, 80, 160 and 320 ppb. The standard solution was then derivatized as described for the fortified milk samples hereafter.

UHT whole cow's milk samples were obtained from a local market in Izmir; Turkey. The milk samples were stored at − 70 °C until analyzed. Working standard solution was used to fortify blank milk samples to obtained milk samples spiked at three levels: 2, 4, 8 ppb.

Centrifugation was performed with refrigerated centrifuge (Hettich Rotina 35R, Boeco, Germany). Strata C_{18}-E (55 μm, 70 A) cartridges of 3 ml and 500 mg (Phenomenex, USA) was used for SPE. Milk samples were analyzed using a Thermo-Electron Corporation LC system (Thermo Scientific, USA). It equipped with Finnigan

Surveyor LC pump coupled with Finnigan Surveyor Photodiade Array UV Detector (PAD) and Finnigan Surveyor Autosampler was used for all analyses. It consists of Synergi 4μm Fusion-RP 80A (150 x 2.00 mm) (Phenomenex, USA). Mobile phases are phosphate buffer, pH 6.5 (Mobil A) and acetonitrile (Mobile B). The gradient system consisted of Mobil A (64%) and Mobil B (36%). The column flow rate was 0.250 ml min^{-1}, the column temperature was 30 °C and the sample injection volume was 100 μl. UV absorbance was measured at 325 nm.

5.2.12.1.2. LC Procedure

The procedure for determination of Penicillin G residues in UHT cow's milk was basically the same as that reported by Verdon and Couder (1998). Spiked milk samples of 5 ml were placed into 50 ml centrifuge glass tubes. 30 ml of phosphate buffer extraction solution (pH 8) were added followed by 780 μl of sulfuric acid to reach pH between 4.0 and 4.5 and the solutions were vortex-mixed for 30 s. These solutions were centrifuged at 4000 rpm for 10 min (3 °C), and were then transferred to clean glass tubes being careful to avoid cream pieces. 565 μl of sodium hydroxide were added to reach pH 8. The aqueous phases were stirred by vortexing and centrifuged again at 4000 rpm for 10 min (3 °C). Solvent reservoirs of 50 ml were mounted onto the C_{18} cartridges and placed with adapters on the SPE vacuum manifold. The cartridges were washed with 10 ml methanol followed by 10 ml water, 5 ml 2% sodium chloride solution and finally with 5 ml phosphate buffer extraction solution pH 8. The flow-rate was not allowed to exceed about 3 ml/min. After this stage, 1 ml volumes of the elution solution (50:50) were poured into the cartridges and for 1 min were allowed to soak homogeneously the C_{18} phase of the cartridges. The Penicillin G was eluted at a flow rate of about 3 ml/min. 0.5 ml of derivatizing reagent were added to the eluate. The glass tubes were stirred and placed into a 65 °C water bath to react about 10 min. The tubes were removed from the water bath, quickly cooled to room temperature for 10 min. If it was protected from light, the derivatized penicillins were stable for 24 h at 4 °C and for more than 5 h at 30 °C. 100 μl of the derivatized sample was injected into HPLC system.

5.2.12.2. Liquid Chromatography Detection of Ampicillin in Milk

5.2.12.2.1. Reagents and Instrumentation for LC Analysis

Ampicillin reference standard was purchased from Riedel-de-Haën (Sigma-Aldrich GmbH Quality Assurance, Germany). Standard stock solution of ampicillin at 2000 ppm were prepared in water and stored at 4 °C for up to 1 month. Intermediate standard solutions of 40 ppb were prepared by dilution of stock solutions with water. Working standard solutions of different concentrations (2, 4, 8, 16 ppb) were prepared daily by diluting with water.

Trichloroacetic acid (TCA), citric acid, formaldehyde, sodium dihydrogenphosphate dihydrate, disodium hydrogenphosphate dihydrate, acetonitrile, acetonitrile hipersolv and methanol hipersolv were purchased from Merck (Darmstadt, Germany). Sodium thiosulfate pentahydrate was purchased from Carlo Erba (Milan, Italy). All chemicals and reagents were of LC grade and analytical grade. Ultra pure water for LC analysis was obtained with an ultra filtration unit system (ELGA Labwater, UK).

The aqueous phosphate buffer for LC analysis was prepared. It involved in 3.4 mM $Na_2HPO_4.2H_2O$, 6.5 mM $NaH_2PO_4.2H_2O$ and 15.7 mM $Na_2S_2O_3.5H2O$. The formaldehyde solution was formaldehyde (7%, v/v) in 0.4 M aqueous citric acid solution.

To establish calibration curves (Figure C.1 in App. C), ampicillin standard solution was diluted with water to prepare calibration standard solutions of different concentration (2, 4, 8, 16 ppb). A 500 µl of TCA (20%, w/v, in water) solution and 500 µl aliquot of formaldehyde (7%, w/v, in water) solution were added to each tube. The tubes were vortexed for 20 s and then heated in a water bath 95 °C for 30 min. After cooling to room temperature, the content in each tube were bought to 2 ml with 20% acetonitrile in water. A 25 µl aliquot of each standard solution was injected into LC for analysis. The peak areas were used for construction of the calibration.

UHT whole cow's milk samples were obtained from a local market in Izmir, Turkey. The milk samples were stored at – 70 °C until analyzed.

Centrifuge was a GR 4.11 refrigerated centrifuge (Jouan, France). The LC system consisted of a SP8800 LC pump and pump controller (Spectra Physics, USA),

RF-10 A XL scanning fluorescence detector (Shimadzu Scientific Instruments, USA) with the excitation wavelenght set at 346 nm and the emission wavelength set at 425 nm and 5μm Aqilent Hypersil C18 250 mmx4 mm column. The coloum temperature was maintained at 30 °C. The LC eluents were: (A) phosphate buffer, (B) acetonitrile (ACN). The isocratic system consisted of 0.05 M KH_2PO_4 at pH 3.0 (77%) and ACN (23%). The flow rate was 0.7 ml min^{-1} and the sample injection volume was 25 μl.

5.2.12.2.2. LC Procedure

The procedure for determination of Ampicillin residues in UHT cow's milk was basically the same as that reported by Ang and Luo (1997). Each milk samples (blank and fortified samples) were deproteinized with 0.5 ml of trichloroacetic acid solution (20%) and 0.5 ml of acetonitrile. Ampicillin residues were extracted into the clear liquid phase, after mixing and centrifugation. The extracts were reacted with trichloroacetic acid and formaldehyde solutions at 95 °C for 30 min to form fluorescent derivatives, which were then determined by LC with fluorescence detection.

5.2.12.3 Liquid Chromatography Detection of Tetracycline in Milk

5.2.12.3.1. Chemicals and Apparatus for LC Analysis

Tetracycline reference standard was obtained from Riedel-de-Haën (Sigma Aldrich GmbH Quality Assurance, Germany). Methanol, acetonitrile, oxalic acid, citric acid monohydrate, disodium hydrogenphosphate dihydrate were LC-grade and were purchased from Merck. Trichloroacetic acid (TCA) and ethylendiaminetetraacetic acid disodium salt (EDTA) were reagent-grade and obtained from Prolab. Ultra pure water for LC analysis was obtained with an ultrafiltration unit system (ELGA Labwater, UK). Solid-phase extraction (SPE) Strata C18-E, 55 μm; 70A (500 mg, 3 ml) were obtained from Phenomenex, USA.

McIlvane buffer was used for the precipitation of protein and extraction of tetracyclines from milk samples. A Na_2EDTA-McIlvaine buffer solution (pH 4) consist of 13.72 g of disodium hydrogen phosphate dihydrate, 11.8 g of citric acid monohydrate and 33.62 g of ethylenediaminetetraacetic acid disodium salt diluted in 1 liter of water.

UHT whole cow's milk samples were obtained from a local market in Izmir; Turkey.The milk samples were stored at – 70 °C until analyzed.

Milk samples were analyzed using a Thermo-Electron Corporation LC system (Thermo Scientific, USA). It equipped with Finnigan Surveyor LC pump coupled with Finnigan Surveyor Photodiade Array Detector (PAD) and Finnigan Surveyor Autosampler was used for all analyses. It consists of Phenomenex C18 Nucleosil column (250 x 3.2 mm, 5 μm). Centrifugation was conducted with a GR 4.11 refrigerated centrifuge (Jouan, France). Mobile phases are 0.01 M oxalic acid (Mobil A) and acetonitrile (Mobile B). The gradient system consisted of Mobil A (80%) and Mobil B (20%). The column flow rate was 0.7 ml min^{-1}, the column temperature was 35 °C and the sample injection volume was 100 μL. UV absorbance was measured at 360 nm.

5.2.12.3.2. LC Procedure

Stock standard solution of tetracycline at 2000 ppm was prepared in water and stored at 4 °C. These solutions were diluted to give a series of working solution (200 ppm, 20 ppm, 2 ppm, 200 ppb, and 10 ppb) that were prepared daily.

Linearity of the detector response was verified with tetracycline standard solution over the range of 250, 500, 750 and 1000 ppb. Calibration curve was prepared daily and estimates the amount of the analytes in milk samples was interpolated from this graphs (Figure C.6 in App. C).

The procedure for determination of tetracycline residues in UHT cow's milk was basically the same as that reported by Cinquina, et al. (2003). 5 ml of milk was homogenised, placed in centrifuge tube and 2 ml of 20% trichloroacetic acid (TCA) added. The milk samples was shaken for 3 min, 20 ml of McIlvaine buffer (pH 4) added and the mixture centrifuged at 4000 rpm for 30 min. The supernatant was then applied to SPE Strata C18-E, previously activated with 3 ml of methanol and 3 ml of water. After sample loading, the cartridge was washed with water and finally tetracycline was eluted with 3 ml of acetonitrile. The solvent was removed under a nitrogen stream at 39-40 °C and the tetracycline residue was dissolved in 2.5 ml of methanol and filtered with 0.45 μm PTFE filter. The sample volume injected in LC system was 100 μl.

5.3. Data Analysis

5.3.1. Statistical Analysis

The statistical analyses were performed using the statistical software Minitab Statistical Software 14 Trial version (Minitab Inc., State College, PA, USA). EC results of milk samples were expressed as the mean and standard deviation. All EC measurements were done five times. One-way analysis of variance (ANOVA) with Fisher's, individual error rate, test was carried out in order to evaluate the effect of the concentration of antibiotic in milk samples at the level of $p < 0.05$. In other words, these tests were performed for all experimental runs to determine significance at 95 percent confidence interval.

For DSC and Lactostar measurements, significant differences between mean values were determined using Fisher's, individual error rate, test following one-way ANOVA. The effect of four selected concentration levels of antibiotics (Penicillin G, Ampicillin, and Tetracycline) compared.

The p-value is the smallest level of significance. It would lead to rejection of the null hypothesis H_0. The ANOVA is a general and one of the most powerful statistical methods that can be used to test the hypothesis that means among two or more groups are equal under the assumption that sampled populations are normally distributed. The reason for doing an ANOVA is to see if there is any difference between group on the same variable. In one-way or one-factor ANOVA, there is only one factor, and the analysis of variance is used to analyze the effect of one factor. The ANOVA table includes the sum of squares, the mean square and an F distribution with degrees of freedom. If the decision is to reject the null, then at least one of the means is different. However, the ANOVA does not tell where the difference lies. For this, another test such as Fisher's test is needed (Montgomery 2001).

5.3.2. The Probabilistic Neural Network (PNN) Method

A design procedure for a novel application of neural networks has been developed to confirm the EC results of this study. This method called the probabilistic neural network (PNN). Probabilistic neural network (PNN) is a statistical algorithm.

PNN is used in solving classification problems and is essentially a nearest-neighbor classifier (Cover and Hart 1967). Defining an m-component multivariate random vector as $X = [x_1, \cdots, x_m]$ starts a general classification problem. The P populations from which the samples are drawn will be indexed by p, $p = 1, \ldots, P$. The prior probability of an unknown sample being drawn from population p is h_p. The cost associated with misclassifying a sample from population p is c_p. In many applications, prior probabilities, h_p, are taken equal and hence can be ignored. The same is also true for costs, c_p. The problem is finding an algorithm that determines the population from which an unknown sample is taken.

If the probability density function $f_p(X)$ is known for all populations, the Bayes optimal decision rule (Masters 1993) is to classify X into population i if

$$h_i c_i f_i(X) > h_j c_j f_j(X)$$

for all populations j not equal to i.

Unfortunately, the probability density function, $f_p(X)$, is not known. Instead, it is estimated using samples. Parzen (Masters 1993) showed how to estimate a probability density function (PDF) from a random sample. As the sample size increases estimated density function gets close to the true density function. The weight function used in estimating the PDF has its largest values for small distances and decreases rapidly toward zero as the distance increases. A common choice for the weighting function is the Gaussian function. The estimator of PDF using the Gaussian weighting function is

$$g_{EST}(x) = \frac{1}{(2\pi)^{p/2} \sigma^p n} \sum_{i=0}^{n-1} e^{-\frac{\|x - x_i\|^2}{2\sigma^2}} \tag{5.2}$$

where p is the number of components of the multivariate sample vector. Before the values are plugged into the above equation, those values need to be normalized to equalize the contribution of each variable. In the field of PNN, estimating the σ is the problem at hand. If σ is chosen too big, then the populations overlap. On the other hand, if σ is chosen too small, then the effect of neighbors in a cluster will be eliminated.

Examining the above equation, the factor π will be the same for all populations. Thus, for PNN it can be ignored. Since the same σ is used for all populations, the factor

in front of the sum that involves σ can be ignored. The above equation will be simplified as

$$g^{'}(x) = \frac{1}{n}\sum_{i=0}^{n-1} e^{-\frac{\|x-x_i\|^2}{\sigma^2}}$$

(5.3)

The equations will be used in PNN to decide whether milk samples are positive (antibiotic containing sample) or negative (free from antibiotic). Figure 5.8 indicates statistical evaluation of the EC data by using PNN method.
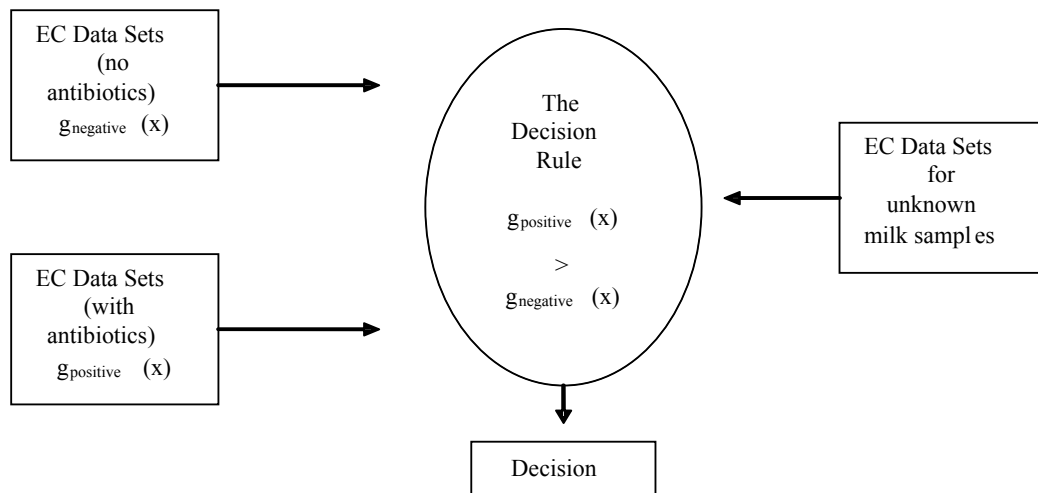

Figure 5.8. EC data by using PNN method

CHAPTER 6

RESULTS AND DISCUSSIONS

6.1. Introduction

UHT whole cow's milk samples fortified with different antibiotics (Penicillin G, Ampicillin and Tetracycline) were tested for determination of antibiotic residues. Milk samples were collected from 2006 to 2008. Milk samples of the same brand were purchased from the same source. It is important that there is no available adequate published information on the status of antibiotic residues in milk and milk product in Turkey. Moreover, there are no efficient antibiotic residue control programs, but there is a requirement to determine management practices at dairy farms. If good management practices are not implemented sufficiently, the health and financial risk involved in antibiotic residues in milk will continue to exist in Turkey. There is also no any satisfactory Residue Database or monitoring program, monitoring and controlling residue to indicate risk to consumer, in Turkey. As before mentioned, antibiotic residue analysis involves both screening and confirmatory methods. This study first focuses on the evaluation of different tests for determining antibiotic residues in milk samples. These tests are Copan Milk Test (microbial inhibitor test with spores of *Bacillus stearothermophilus* var. *calidolactis*, ROSA Test (receptor assay) and Penzyme Milk Test (enzymatic assay). In antibiotic residue screening of milk, the antibiotic residue tests may be over-sensitive for the MRL values for certain antibiotic residues. Therefore, samples may be determined as positive when antibiotic residues lower than the MRL. This may be a problem result. It leads to a false positive or false negative, especially at lower levels. These false results may cause enormous economic losses. In order to overcome this problem associated with test kits, milk samples were artificially contaminated with selected concentrations of Penicillin G, Ampicillin and Tetracycline. The residue test kits and the selected concentration of target antibiotics were analyzed by using confirmatory methods. These confirmatory methods are based on HPLC analysis. In addition to these analyses, physical, thermo-physical and chemical properties of milk samples were determined to reveal whether these measurements can

indicate any differences between antibiotic free milk (control) and milk containing different levels of Penicillin G, Ampicillin and Tetracycline.

6.2. Initial Screening of Milk Samples

Before the all measurements, the initial screening of eight different UHT whole cow's milk samples was examined visually by using Copan Milk Test. After the incubation time, color turned purple or yellow. A purple color indicates a positive result (existence of antibiotics) whereas a color change to yellow indicates a negative result (no antibiotic). These results are illustrated in Figure 6.1. The interpretation is in accordance with the color card for Copan Milk Test. Copan Milk Test showed three of them "positive", three of them "negative" and one of them "partial negative". According to these results, milk samples were further analyzed in order to confirm the existence of any antibiotic residue by yogurt culture test. In other words, yogurt culture test was performed to confirm false-positive outcomes in milk samples. During the yogurt culture test, yogurt formation was not observed at positive samples. Table 6.1 shows Copan Milk Test and yogurt culture test results for UHT whole cow's milk samples. After the Copan Milk Test and yogurt culture test, one negative milk sample was chosen for the measurements and fortified with Penicillin G, Ampicillin and Tetracycline at the selected concentrations.

Figure 6.1. Visual interpretation of Copan Milk Test result

Table 6.1. Copan milk test and yogurt culture test results for milk samples

Milk Samples	Copan Milk Test (reading at 3 h +/- 15')		Yogurt Culture Test
	Visual Reading	Color Card for Copan Milk Test	Yogurt Formation
Sample 1	Yellow	Negative	Yogurt Formation (+)
Sample 2	Yellow	Negative	Yogurt Formation (+)
Sample 3	Yellow	Negative	Yogurt Formation (+)
Sample 4	Yellow + Purple	Partial Negative	Not a good characteristic curd for yogurt (+/-)
Sample 5	Purple	Positive	No yogurt Formation (-)
Sample 6	Purple	Positive	No yogurt Formation (-)
Sample 7	Purple	Positive	No yogurt Formation (-)
Sample 8	Purple	Positive	No yogurt Formation (-)

6.3. The Acidity, pH and Density of Milk Samples

Firstly, milk samples were tested by Copan Milk Test. This test results indicated that milk samples did not contain antibiotic residue. Then milk samples were artifically contaminated with MRL level of Penicillin G, Ampicillin and Tetracycline.

After that procedure, the acidity, pH and density of four antibiotic free milk samples and spiked samples were measured at 25 °C. The acidity and pH results of milk samples are presented in Table 6.2. The acidity and pH of antibiotic free milk samples and spiked milk samples were determined similar value with each other. In addition, the acidity and pH results of milk samples showed similar result with literature. The titratable acidity of healthy cow milk is in the range of 0.14% - 0.16% in terms of milk acid (Metin 2001 and Rosenthal 1991). pH of bovine milk is in the range of 6.22 - 6.77 (Jensen 1995). Characteristic pH value at 25 $^{\circ}$C is measured as 6.6 and reported range is 6.5-6.7 (Webb, et al. 1974).

Table 6.2. Acidity and pH results of milk samples

Milk Sample	Acidity (% lactic acid)	pH (25 °C)
Sample 1	0.1450±0	6.650±0
Sample 1 containing antibiotic	0.1425±0.0035	6.650±0
Sample 2	0.1475±0.0035	6.585±0.0071
Sample 2 containing antibiotic	0.1475±0.0035	6.600±0
Sample 3	0.1300±0	6.660±0
Sample 3 containing antibiotic	0.1300±0	6.665±0.0071
Sample 4	0.1550±0	6.545±0.0071
Sample 4 containing antibiotic	0.1500±0	6.520±0.0141
Literature	0.14 - 0.16	6.4 - 6.7

Table 6.3, Table 6.4, Table 6.5 and Table 6.6 indicated that there are no differences between density values of control and spiked samples.

Figure 6.2, Figure 6.3, Figure 6.4 and Figure 6.5 show data obtained for milk samples having at least 3.1% fat content at different temperatures (15, 20, 25, 30, 35, 40, 45, 50 °C, respectively). Similar behavior was obtained for both antibiotic free milk samples and spiked milk samples. It was found that the density of milk samples decreased with increasing temperature for all milk samples.

These results indicated that heat treatment had significant effect on the density of milk samples. One of the aspects of the density of milk is the effects of temperature. Mean density of milk samples ranges from 1.03463 at 0 °C to 1.02092 at 44 °C. The mean density is 1.3071 at 18 °C (Rutz et al. 1955). Density is so changeable due to all substances contributing to its composition. The density of milk is 1.027 g/ml − 1.035 g/ml at 20 °C based on its composition. It decreases because of increasing fat content and increases because of decreasing fat content. The density of milk also increases because of increasing quantity of protein, lactose and mineral substance. Increasing temperature causes a decrease in density of milk (Metin 2001). According to Neville and Jensen (1995), the specific gravity of milk is 1.021 − 1.037. The density of milk normally ranges from 1.028 and 1.034 depending on composition. That is why the results given here are specific to tested milk samples and show some variability based

on composition differences. Overall, milk is very slightly denser than water and presence of antibiotics of MRL level did not affect the density values of UHT whole milk samples.

Table 6.3. Density results for milk sample 1

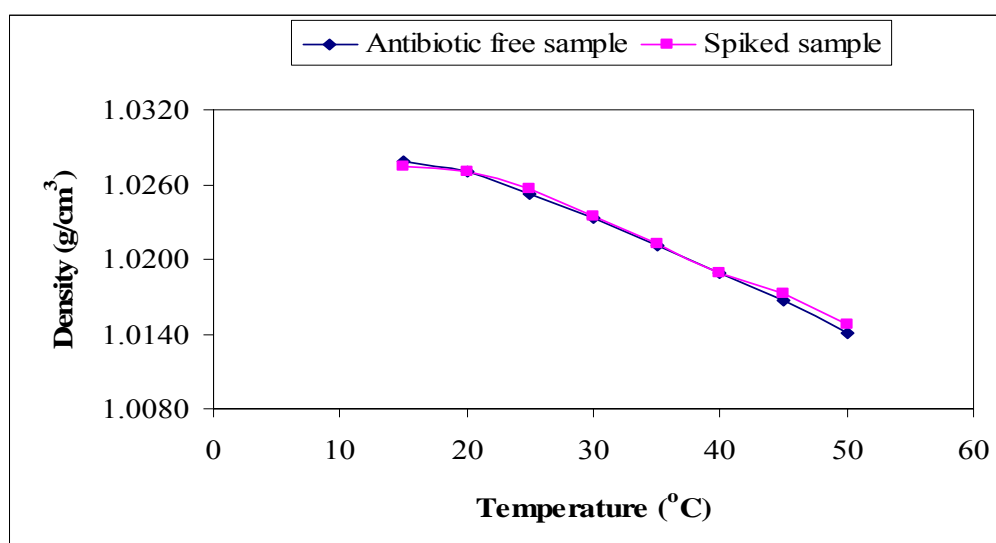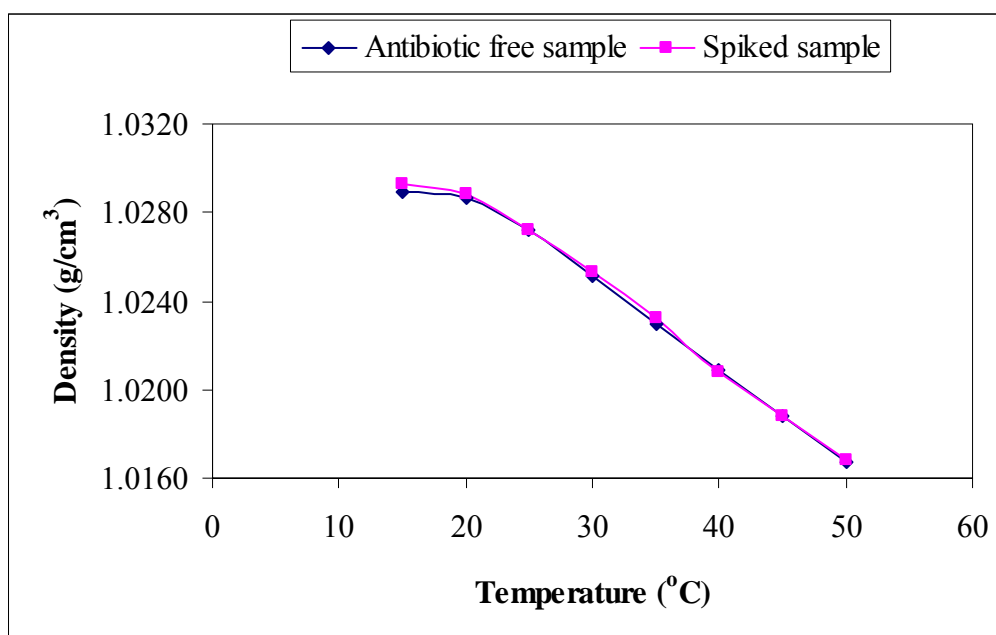| Milk Sample 1	Temperature (°C)	Density (g/cm^3)
Antibiotic free	15	1.0279±0.0002
Antibiotic free	20	1.0271±0
Antibiotic free	25	1.0253±0
Antibiotic free	30	1.0233±0
Antibiotic free	35	1.0211±0.0001
Antibiotic free	40	1.0189±0
Antibiotic free	45	1.0167±0
Antibiotic free	50	1.0141±0.0004
Fortified with antibiotic	15	1.0274±0
Fortified with antibiotic	20	1.0271±0
Fortified with antibiotic	25	1.0256±0
Fortified with antibiotic	30	1.0235±0.0002
Fortified with antibiotic	35	1.0212±0
Fortified with antibiotic	40	1.0189±0
Fortified with antibiotic	45	1.0172±0.0002
Fortified with antibiotic	50	1.0147±0.0002

Figure 6.2. Density change vs temperature for antibiotic free milk sample 1 and spiked milk sample 1

Table 6.4. Density results for milk sample 2

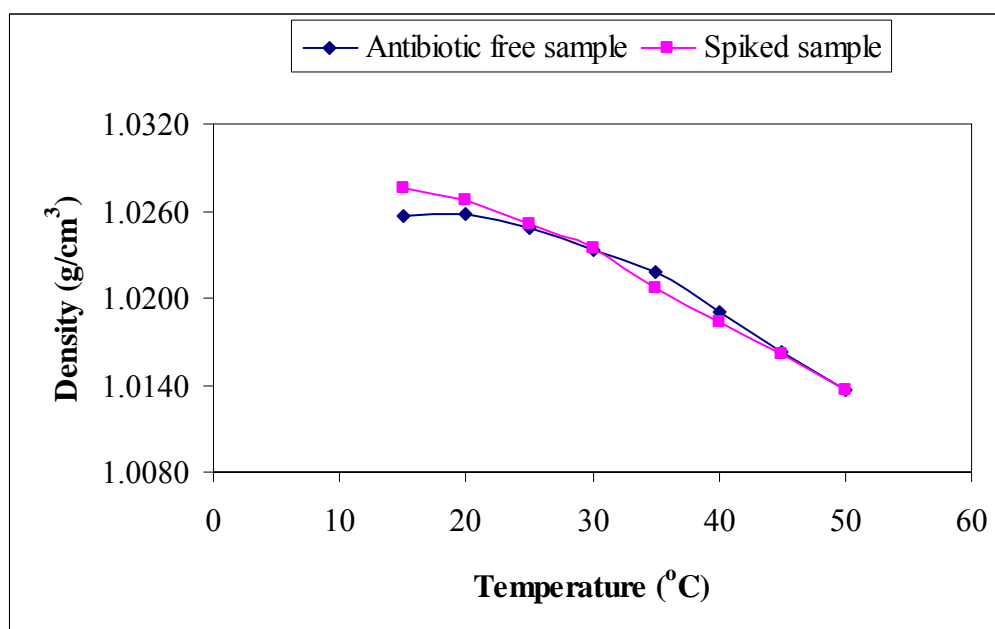| Milk Sample 2	Temperature (°C)	Density (g/cm^3)
Antibiotic free	15	1.0289±0.0001
Antibiotic free	20	1.0287±0
Antibiotic free	25	1.0272±0
Antibiotic free	30	1.0251±0
Antibiotic free	35	1.0230±0
Antibiotic free	40	1.0209±0
Antibiotic free	45	1.0188±0
Antibiotic free	50	1.0167±0
Fortified with antibiotic	15	1.0293±0
Fortified with antibiotic	20	1.0288±0
Fortified with antibiotic	25	1.0272±0.0001
Fortified with antibiotic	30	1.0253±0
Fortified with antibiotic	35	1.0232±0
Fortified with antibiotic	40	1.0208±0
Fortified with antibiotic	45	1.0188±0.0001
Fortified with antibiotic	50	1.0168±0

Figure 6.3. Density change vs temperature for antibiotic free milk sample 2 and spiked
milk sample 2

Table 6.5. Density results for milk sample 3

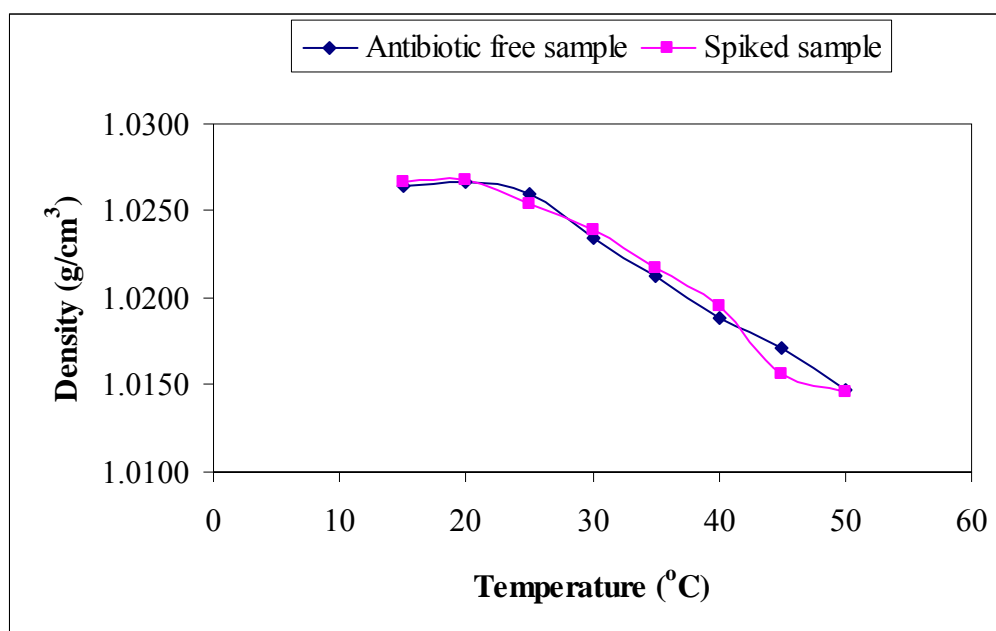| Milk Sample 3	Temperature (°C)	Density (g/cm^3)
Antibiotic free	15	1.0256±0
Antibiotic free	20	1.0258±0
Antibiotic free	25	1.0248±0
Antibiotic free	30	1.0233±0
Antibiotic free	35	1.0218±0
Antibiotic free	40	1.0190±0
Antibiotic free	45	1.0163±0.0002
Antibiotic free	50	1.0136±0.0001
Fortified with antibiotic	15	1.0276±0
Fortified with antibiotic	20	1.0268±0
Fortified with antibiotic	25	1.0251±0
Fortified with antibiotic	30	1.0235±0
Fortified with antibiotic	35	1.0207±0
Fortified with antibiotic	40	1.0184±0.0001
Fortified with antibiotic	45	1.0162±0
Fortified with antibiotic	50	1.0137±0

Figure 6.4. Density change vs temperature for antibiotic free milk sample 3 and milk sample 3 containing antibiotic

Table 6.6. Density results for milk sample 4

Milk Sample 4	Temperature (°C)	Density (g/cm^3)
Antibiotic free	15	1.0264±0.0001
Antibiotic free	20	1.0267±0
Antibiotic free	25	1.0260±0
Antibiotic free	30	1.0235±0
Antibiotic free	35	1.0213±0
Antibiotic free	40	1.0189±0
Antibiotic free	45	1.0171±0
Antibiotic free	50	1.0147±0
Fortified with antibiotic	15	1.0267±0.0002
Fortified with antibiotic	20	1.0268±0
Fortified with antibiotic	25	1.0254±0.0002
Fortified with antibiotic	30	1.0239±0
Fortified with antibiotic	35	1.0217±0.0002
Fortified with antibiotic	40	1.0195±0
Fortified with antibiotic	45	1.0156±0.0002
Fortified with antibiotic	50	1.0146±0

Figure 6.5. Density change vs temperature for antibiotic free milk sample 4 and milk sample 4 containing antibiotic

6.4. The Electrical Conductivity of Milk Samples

Prior to electrical conductivity (EC) measurements, The Copan Milk Test, Penzyme and Rosa Test, having different principle of method were evaluated for determining antibiotic residues in selected brand of UHT whole cow's milk samples. The results of test kits indicated that no antibiotic was present in these samples. The Figure 6.6 shows these results visually. The visual results of the milk tests were also confirmed yogurt culture test. After these results, electrical conductivity measurements were performed to detect the antibiotics in these samples. For this purpose, Penicillin G, Ampicillin and Tetracycline were chosen target antibiotic in this study. Milk samples were artificially contaminated with selected concentrations of these antibiotics. Electrical conductivity measurements were conducted both on the whole milk fortified with Penicillin G, Ampicillin, Tetracycline and on whole cow's milk free from antibiotics (control). Conductivity data collected along one year interval period for each seasons and for each antibiotics from 2006 to 2008.

Figure 6.6. Visual interpretation of Copan Milk Test, Penzyme and ROSA Test results

Following observations can be drawn out from the usage of different test kits: The Copan Milk Test is new inhibition assay. It was developed more recently and consists of the nutrient pre-corporated in the agar. Penzyme is an enzyme colorimetric assay for rapid determination of beta-lactam antibiotics. ROSA Test is receptor assay. The drawback of Penzyme tests is based on having a rather narrow antibiotic spectrum including only beta-lactams as compared to two other tests. According to testing procedures, the Copan milk test is simple to use, easy to read and having a long shelf life. The ROSA test is a good immuno-receptor test because it is fast to run, simple to use. Despite these advantages, ROSA reader device is required for testing milk samples, because detection can be difficult without the reader. Table 6.7 shows that detection limits of Copan Milk Test and ROSA Test. In contrast to these milk tests, Penyzme Test was used only for detection of beta-lactams. It was not suitable for detection of other antibiotic residues. In contrast to the detection limit of Copan Milk Test, ROSA test is more sensitive for tetracycline residues. However, Copan milk test found more sensitive for detection of beta-lactams.

Table 6.7. Limits of detection with Copan Milk Test and ROSA Test

Milk Test	Antibiotics	Test Detection Limit (ppb)	MRL (ppb)
Copan Milk Test			
	Penicillin G	1 – 2	4
	Ampicillin	< 2	4
	Tetracycline	250 - 500	100
ROSA Test			
	Penicillin G	2 - 3	4
	Ampicillin	3 - 4	4
	Tetracycline	30 - 90	100

Yogurt formation was observed at the end of 6 hours incubation time. Yogurt culture test results for milk samples are given in Table 6.8. The change in the consistency and pH during yogurt formation was also observed for milk samples containing Penicillin G, Ampicillin, and Tetracycline at selected concentrations. pH of milk samples was measured at 0., 2., 4., 6. hour of incubation time. The blank sample (antibiotic free milk) reached the maximum curd firmless at about pH 4.6 due to the fact that sufficient acidity was generated at the end of 6 h to form a coagulum. Different

concentrations of Penicillin G, Ampicillin and Tetracycline gave no curd formation results. pH of the antibiotic positive milk samples was determined to have the highest pH level in contrast to negative milk samples. Yogurt formation was not observed at and above 2 ppb Penicillin G and Ampicillin concentration, and at and above 100 ppb Tetracycline concentration.

Table 6.8. Consistency and pH of milk samples containing selected concentrations of Penicillin G, Ampicillin, Tetracycline

	Concentration (ppb)	pH (at the end of incubation time)	Consistency (visually)
Penicillin G			
	0	4.487±0.1224	Custard-like curd
	2	5.707±0.1490	No Yogurt Formation
	4	5.766±0.1188	No Yogurt Formation
	8	5.974±0.1203	No Yogurt Formation
Ampicillin			
	0	4.578±0.2079	Custard-like curd
	2	6.428±0.1303	No Yogurt Formation
	4	6.444±0.1165	No Yogurt Formation
	8	6.465±0.1036	No Yogurt Formation
Tetracycline			
	0	4.470±0.2026	Custard-like curd
	100	5.583±0.2042	No Yogurt Formation
	250	5.854±0.1805	No Yogurt Formation
	500	6.037±0.1203	No Yogurt Formation
Literature	0	~ 4.600	The maximum curd

According to Grunwald and Petz (2002), coagulation was influenced negatively by increasing concentrations of antibiotic residues. The precipitation of milk proteins and the impact of living yoghurt cultures on penicillin residues result in the decreasing

pH. The polarity of the side chain of the penicillin (ampicillin < penicillin) decreased with increasing the amount of bound penicillins. These might be indicated by preferred binding to hydrophobic sites in the milk protein.

After the Copan, Penzyme, ROSA and yogurt culture tests, negative milk samples were chosen for the electrical conductivity measurements and fortified with Penicillin G, Ampicillin and Tetracycline at the selected concentrations.

Electrical conductivity of antibiotic free milk samples is changed from 5.285 to 5.341 mS/cm at 25 °C. The typical electrical conductivity of milk at 25 °C is reported as 4.0 - 5.5 mS/cm (Therdthai and Zhou 2001). It was observed that the electrical conductivity of milk samples increased slightly with increasing antibiotic concentration, as can be seen from Figure 6.7. According to Norberg (2005), EC of the milk samples increases because of increasing concentration of Na^+, K^+ and Cl^- in milk. The evaluating conductivity at Penicillin G, Ampicillin and Tetracycline at selected concentrations demonstrates that it is of significance for having knowledge of how concentrations of antibiotics affect the electrical conductivity of milk.

According to Zhuang, et al. (1997) and Mucchetti, et al. (1994), the electrical conductivity of milk is especially due to its mineral salt fraction and the protein content is not of major importance. Lactose is an uncharged sugar thereby it does not conduct current. Fat is a nonconductor. This means it hinders the conduction of electricity by reducing volume and by impeding the mobility of ions. Because of the thin nonconductive membrane that covers the fat globules, EC decreases with increasing fat content in milk (Norberg 2005). Casein shows a very low conductivity in contrast to milk salts. Hence, the conductivity of milk is determined primarily by H^+ and Cl^- and also other ions. UHT whole milk has higher electrical conductivity than unprocessed milk because of the reduction in size of the milk fat globules. In addition, EC of milk affects by mastitis.

The electrical conductivity of milk samples changes in terms of mastitis, the season, the age of milk, milking interval, the stage of lactation, the breed of cattle and milk composition (Norberg 2005). It is indicated that these factors affect conducting ions in milk. Therefore, UHT whole cow's milk samples of the same brand were purchased from the same source. The effect of seasonal differences on electrical conductivity was also investigated Figure 6.7. The milk samples were collected and tested at every month during the autumn, winter, spring and summer periods starting in August 2006.
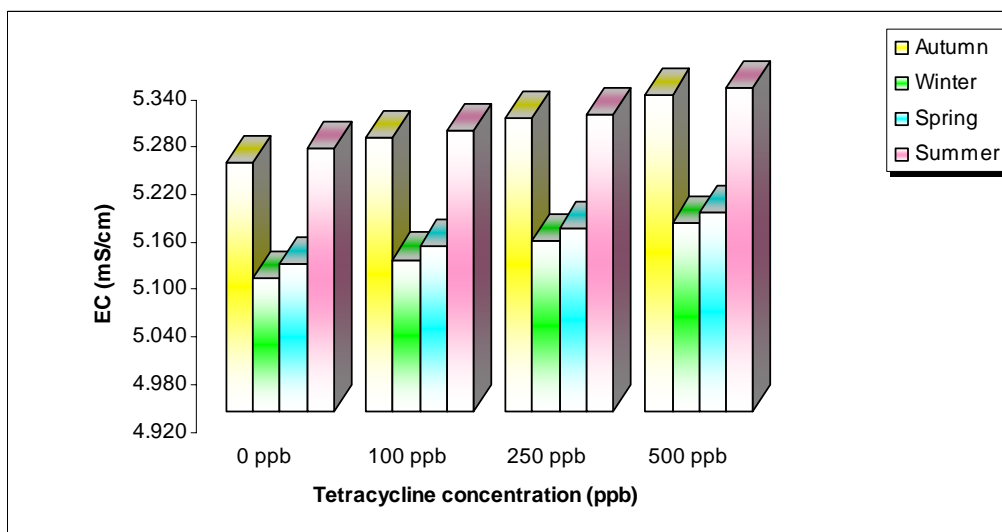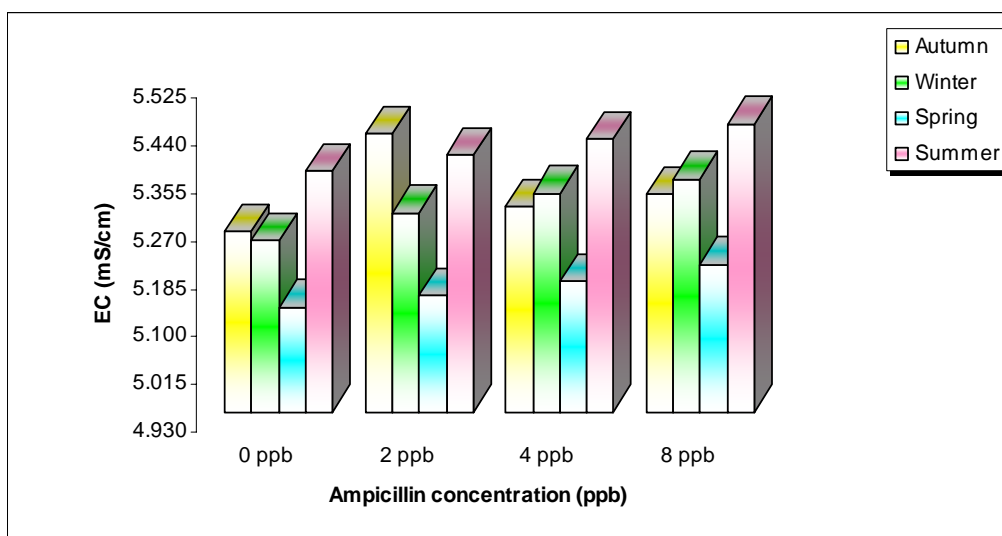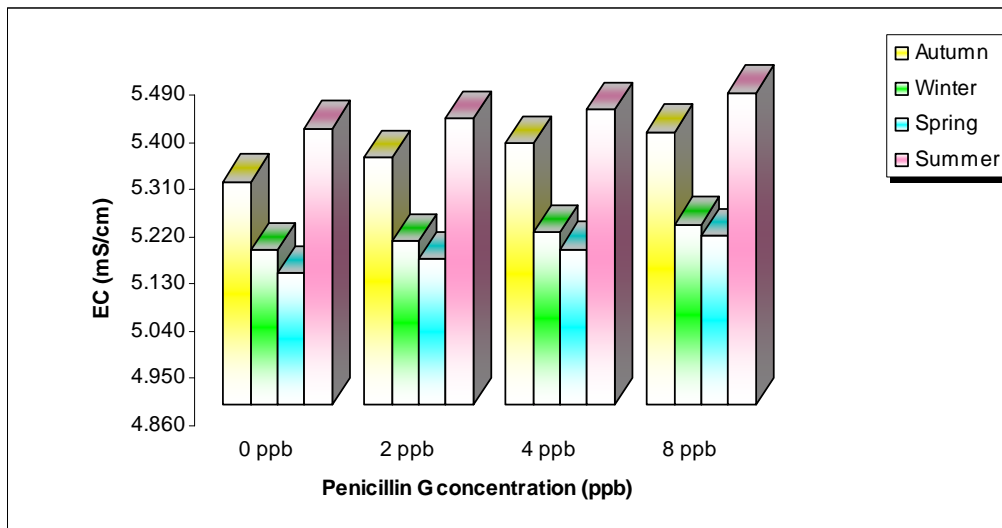
Figure 6.7. EC of milk samples containing selected concentrations of Penicillin G, Ampicillin and Tetracycline (samples collected at different periods of the year)

EC of milk has been commonly used for monitoring mastitis for many years. Therefore, the differences in electrical conductivity of antibiotic free milk sample and the milk samples containing Penicillin G, Ampicillin and Tetracycline at selected concentrations were examined statistically in order to evaluate the EC measurement is or is not a good detection tool for antibiotic residues in milk. One-way ANOVA with Fisher's test, individual error rate, were performed to evaluate the EC results (Table 6.9, Table 6.10 and Table 6.11). The means of antibiotic free milk samples and milk samples containing selected concentrations of Penicillin G, Ampicillin and Tetracycline were assessed. The ANOVA results addressed that the means of milk samples were statistically different at the significant level of 5 % and concluded that there was strong evidence to indicate that the mean of electrical conductivity of milk samples differ from each other ($p<0.05$). The selected concentrations of Penicillin G, Ampicillin and Tetracycline significantly affected the mean of electrical conductivity.

Table 6.9. ANOVA results of EC for milk containing Penicillin G

Penicillin G Concentration (ppb)	Electrical Conductivity (mS/cm)
Autumn	
0	5.2873 ± 0.0799^a
2	5.3327 ± 0.0729^{ab}
4	5.3600 ± 0.0713^{ab}
8	5.3820 ± 0.0766^b
Winter	
0	5.1560 ± 0.0287^a
2	5.1733 ± 0.0253^{ab}
4	5.1907 ± 0.0237^{bc}
8	5.2060 ± 0.0247^c
Spring	
0	5.1133 ± 0.0261^a
2	5.1387 ± 0.0196^b
4	5.1580 ± 0.0251^b
8	5.1833 ± 0.0209^c

Table 6.9 (cont.). ANOVA results of EC for milk containing Penicillin G

Summer	
0	5.3887 ± 0.0245^a
2	5.4067 ± 0.0168^b
4	5.4247 ± 0.0052^b
8	5.4567 ± 0.0140^c

[a-c]values in a column with the same superscript are not significantly different by Fisher' s tests ($p<0.05$)

Table 6.10. ANOVA results of EC for milk containing Ampicillin

Ampicillin Concentration (ppb)	Electrical Conductivity (mS/cm)
Autumn	
0	5.2513 ± 0.0476^a
2	5.2740 ± 0.0503^{ab}
4	5.2960 ± 0.0574^{ab}
8	5.3187 ± 0.0061^b
Winter	
0	5.2347 ± 0.0508^a
2	5.2847 ± 0.0168^b
4	5.3193 ± 0.0276^c
8	5.3447 ± 0.0250^c
Spring	
0	5.1167 ± 0.0403^a
2	5.1380 ± 0.0399^{ab}
4	5.1620 ± 0.0414^{bc}
8	5.1933 ± 0.0497^c
Summer	
0	5.3600 ± 0.0196^a
2	5.3880 ± 0.0211^b
4	5.4153 ± 0.0177^c
8	5.4427 ± 0.0167^d

Table 6.11. ANOVA results of EC for milk containing Tetracycline

Tetracycline Concentration (ppb)	Electrical Conductivity (mS/cm)
Autumn	
0	5.2340 ± 0.0600^a
100	5.2660 ± 0.0680^{ab}
250	5.2907 ± 0.0713^{ab}
500	5.3200 ± 0.0721^b
Winter	
0	5.0893 ± 0.0183^a
100	5.1120 ± 0.0197^b
250	5.1353 ± 0.0151^c
500	5.1580 ± 0.0170^d
Spring	
0	5.1060 ± 0.0253^a
100	5.1293 ± 0.0183^b
250	5.1507 ± 0.0202^{bc}
500	5.1707 ± 0.0209^c
Summer	
0	5.2567 ± 0.0070^a
100	5.27533 ± 0.0052^b
250	5.29533 ± 0.0074^c
500	5.32867 ± 0.0074^d

[a-d]Column means having a different letter are significantly different ($p < 0.05$)

After the promising results obtained from electrical conductivity measurements, PNN method was performed in order to classify unknown milk samples as positive or negative. Electrical conductivity data of negative and positive milk samples spiked with Penicillin G, Ampicillin and Tetracycline were used as input data to PNN and unknown data sets were tested to be classified as whether they were antibiotic positive or negative. Overall, the probabilistic neural network (PNN) achieved an average recognition performance of 100%. This high level of recognition suggests that the PNN

is a promising method for detecting the divergence among the electrical conductivity of different samples.

In other words, the results of this study showed that the electrical conductivity measurement along with PNN method was proven to be used for detection of antibiotic residues in milk but needs to be investigated more in terms of milk composition. It is known that increasing protein concentration increases the EC whereas increasing the lactose and fat concentration decreases the EC. This research is demonstrated that a computer program can be developed by combining PNN method with interaction between EC and milk constituents.

6.5. The DSC Results of Milk Samples

Thermo-physical properties of milk containing antibiotic residues have not been published yet. In this study, melting and evaporation data were used to characterize thermal behavior of antibiotic free milk samples and milk samples fortified with Penicillin G (0, 2, 4, 8 ppb) Ampicillin (0, 2, 4, 8 ppb) and Tetracycline (0, 100, 250, 500 ppb). Melting and evaporation points were recorded at the maximum of endothermic peaks.

Figure 6.8, 6.9 and 6.10 show effects of antibiotic concentration on melting temperature, heat of fusion, evaporation temperature and heat of evaporation of antibiotic free milk samples and milk samples containing Penicillin G, Ampicillin and Tetracycline. Heat of fusion and heat of evaporation and evaporation temperature are increased by increasing antibiotic concentrations. On the other hand, melting temperature is increased at the same conditions.

Figure 6.11, 6.12 and 6.13 indicate DSC. DSC curves of target antibiotics at selected concentrations show big endothermic peaks. As can be seen Figure 6.11, 6.12 and 6.13, there are differences in melting temperature, heat of fusion, evaporation temperature, heat of evaporation and onset temperatures of transitions.

It was observed that the peak temperatures and areas increased with increasing antibiotic concentration levels. Peaks appeared sharper and clearer with the increase of endothermic enthalpy. These differences provide a basis for detection of antibiotic residues in milk using DSC. The statistical testing showed that antibiotic concentration had a significant effect on these thermal parameters (melting temperature, heat of

fusion, evaporation temperature and heat of evaporation) at a 95% confidence level. The Fisher's, individual error rate, test were clearly shown in Table 6.8 for antibiotic free milk samples and milk samples fortified with Penicillin G, Ampicillin and Tetracycline at selected concentrations.

In thermal behavior of antibiotic free milk samples and milk spiked with Penicillin G, Ampicillin, Tetracycline, there are two endothermic peaks for each sample. For Penicillin G residues, the DSC thermo-gram of milk samples shows two endothermic signal around 3.6000 to 1.9850, 112.625 to 129.970 °C, respectively. It is conclude that first thermal event is a melting endotherm and second one is an evaporation endotherm. For Ampicillin residues, first endothermic peak is in the temperature range of 2.9850 to 1.7050 °C. Second endothermic peak is a very wide and intense endothermic peak in the temperature range of 113.380 to 123.565 °C. For Tetracycline residues, the average values of first endothermic peak temperature of milk samples at selected concentrations (0, 100, 250, 500 ppb) is changed from 3.5600 to 2.3050 °C. Second endothermic peak temperature is in between 192.60 to 218.30 °C. All peaks appeared sharper and clearer with the increase of endothermic enthalpy.
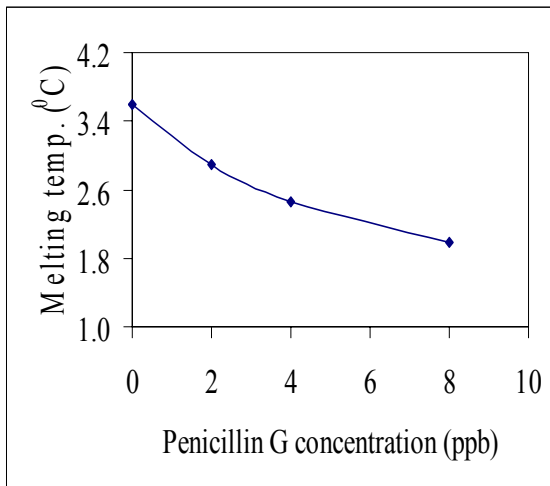
(a)
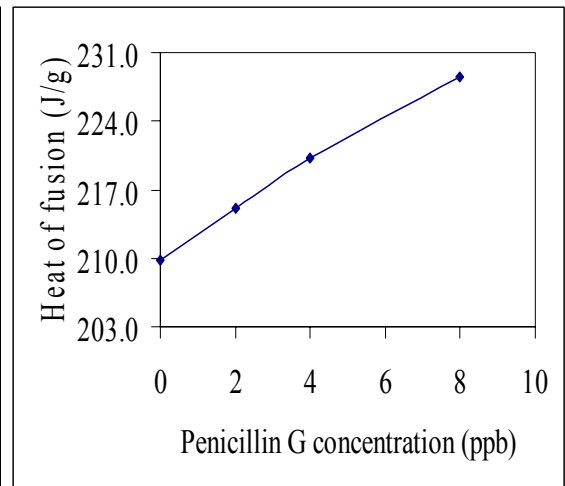
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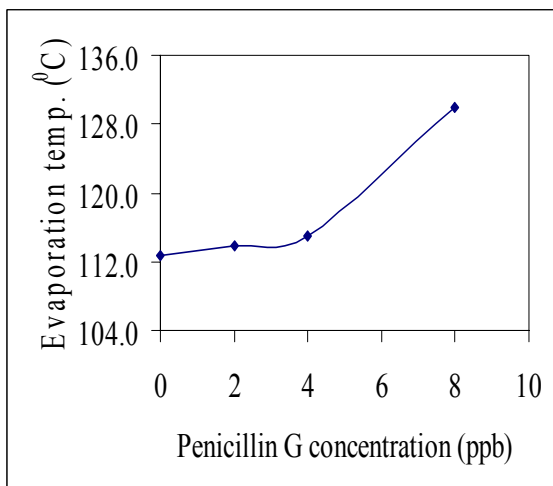
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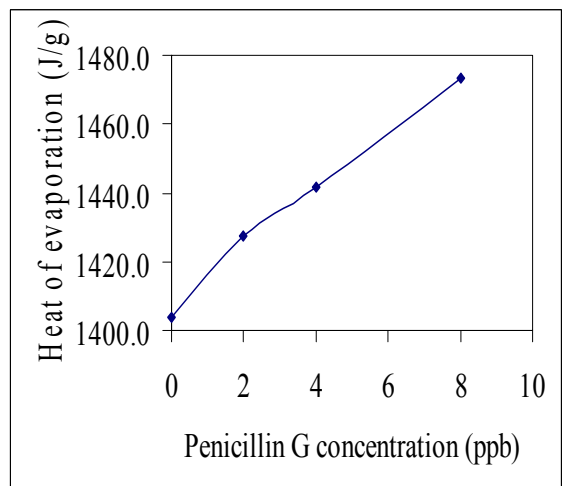
(d)

Figure 6.8. Effects of Penicillin G concentration on the thermal parameters (a) melting temperature, (b) heat of fusion, (c) evaporation temperature, (d) heat of evaporation of milk samples
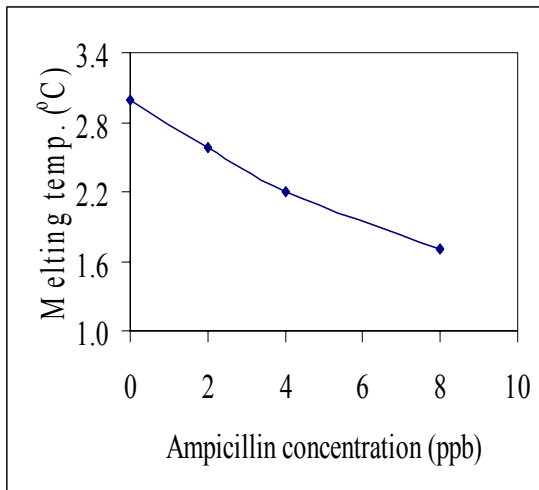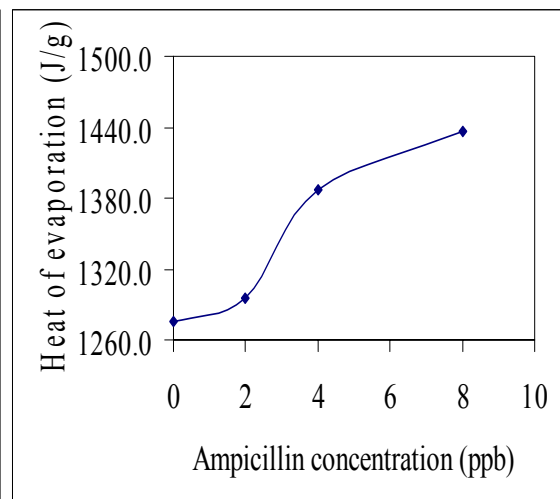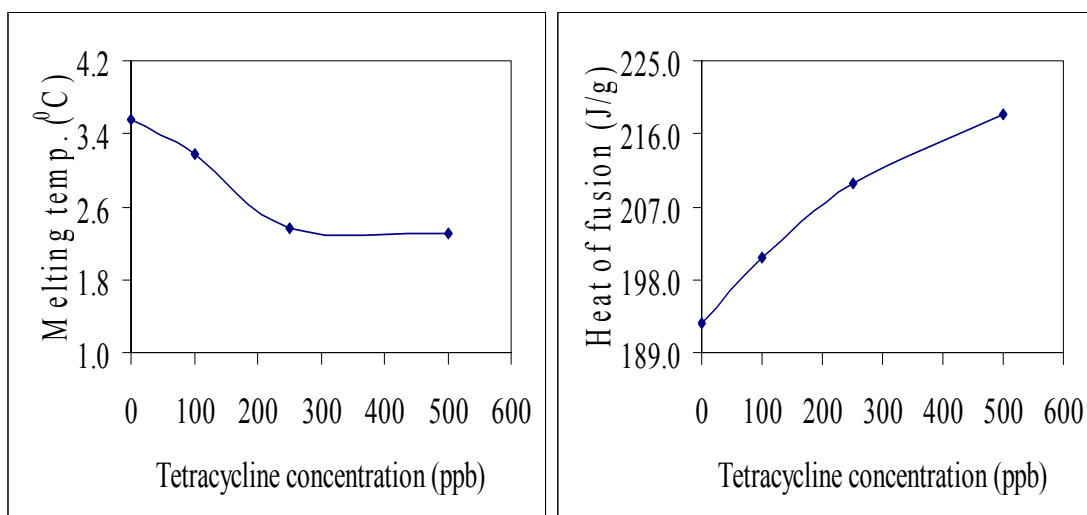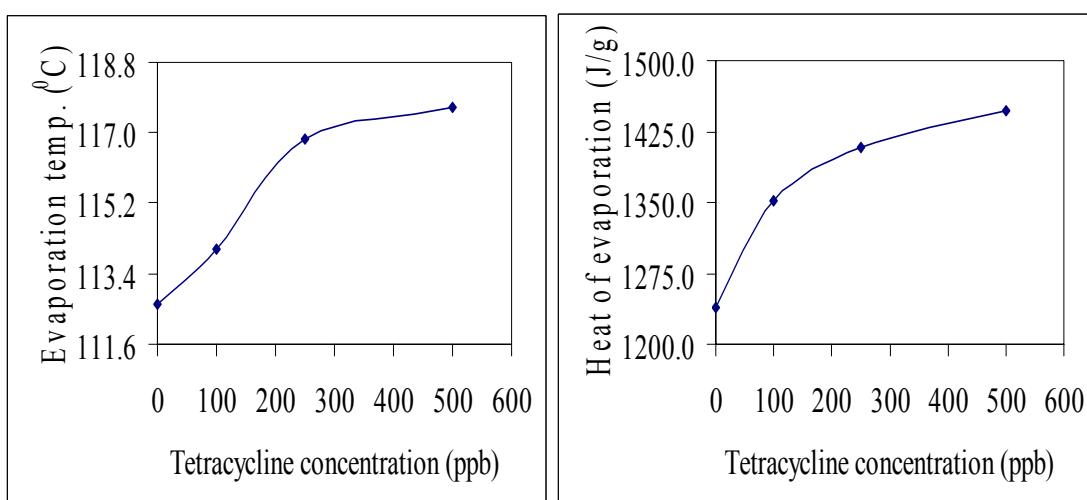
(a)
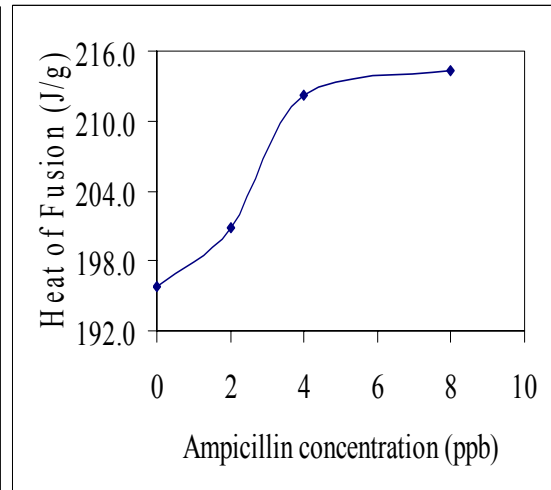
(b)
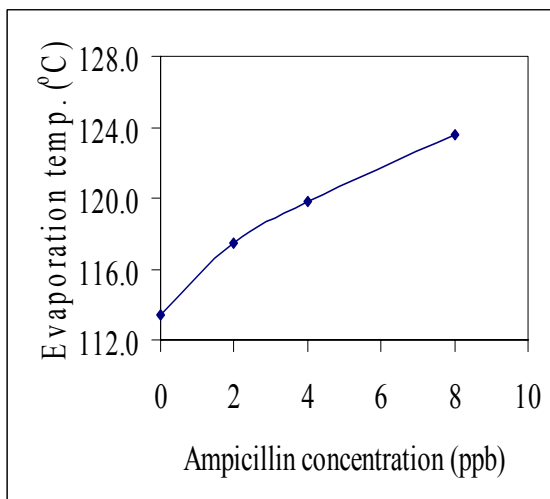
(c)

(d)

Figure 6.9. Effects of Ampicillin concentration on the thermal parameters (a) melting temperature, (b) heat of fusion, (c) evaporation temperature, (d) heat of evaporation of milk samples

Figure 6.10. Effects of Tetracycline concentration on the thermal parameters (a) melting temperature, (b) heat of fusion, (c) evaporation temperature, (d) heat of evaporation of milk samples
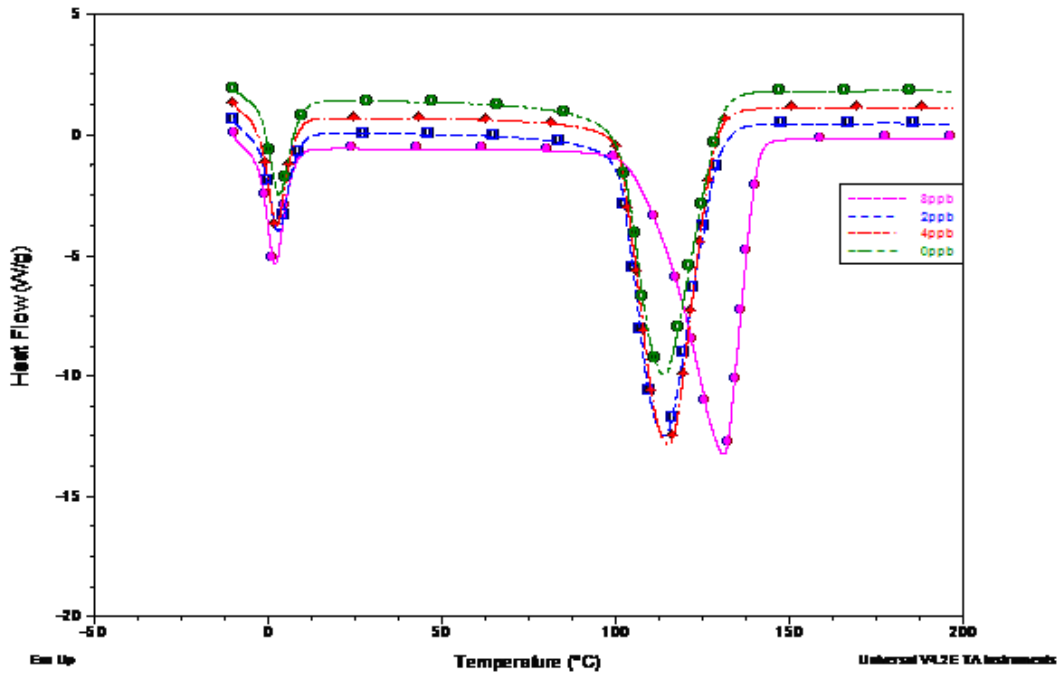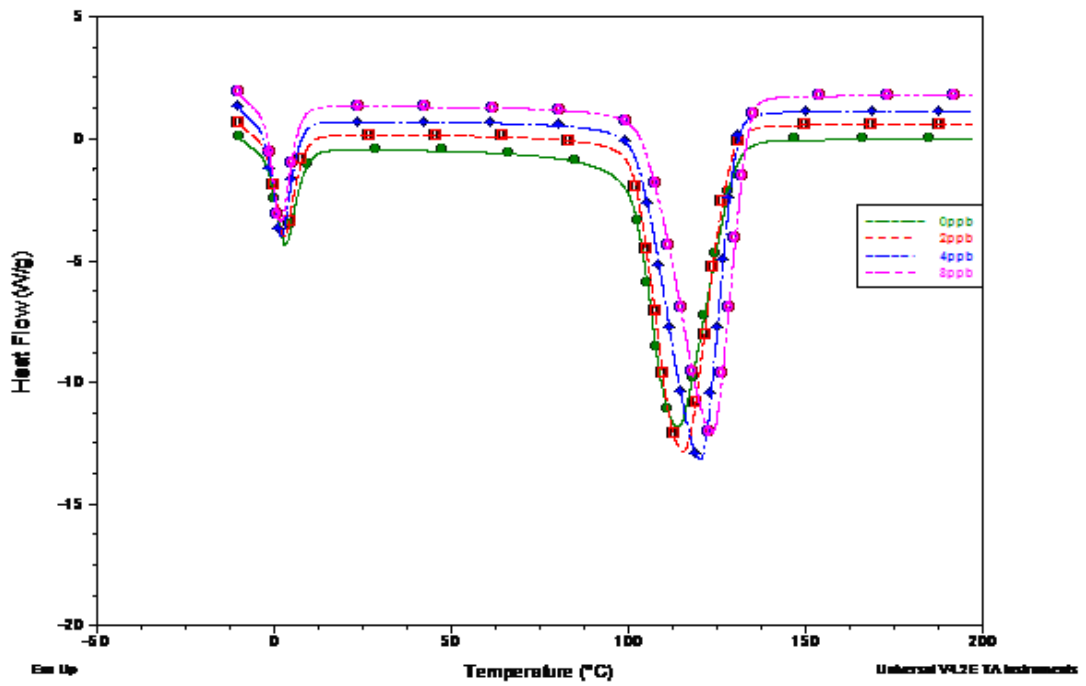
Figure 6.11. DSC curves of milk samples containing 0, 2, 4, 8 ppb Penicillin G

Figure 6.12. DSC curves of milk samples containing 0, 2, 4, 8 ppb Ampicillin
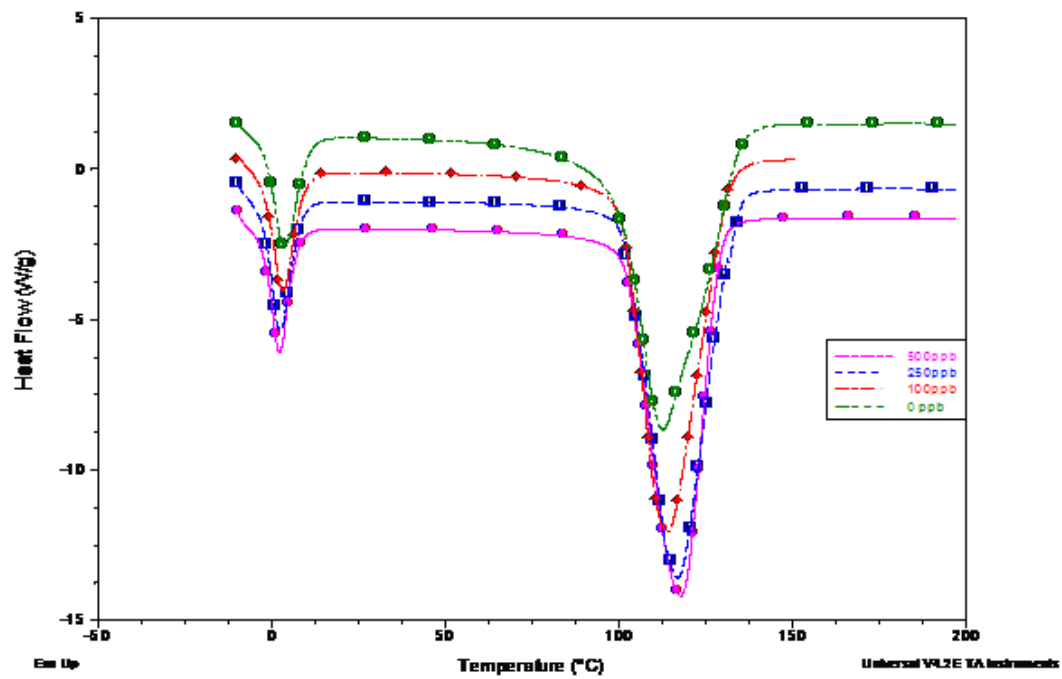
Figure 6.13. DSC curves of milk samples containing 0, 100, 250, 500 ppb Tetracycline

Table 6.12. Influence of antibiotic concentration on the DSC curves of milk samples with results of Fisher's, individual error rate, test

Type of antibiotic	Concentration (ppb)	Peak Temperature (°C)		Peak Area (J g^{-1})	
		1. peak	2. peak	1. peak	2. peak
Penicillin G					
	0	3.6000 ± 0.0707[a]	112.625 ± 0.035[a]	209.80 ± 2.40[a]	1404.00 ± 18.38[a]
	2	2.8950 ± 0.0495[b]	113.770 ± 0.042[a]	215.10 ± 0.42[b]	1427.50 ± 0.71[ab]
	4	2.4500 ± 0.0849[c]	114.940 ± 0.014[a]	220.20 ± 0.42[c]	1441.50 ± 6.36[bc]
	8	1.9850 ± 0.1061[d]	129.970 ± 1.513[b]	228.45 ± 0.64[d]	1473.50 ± 0.71[c]
Ampicillin					
	0	2.9850 ± 0.0212[a]	195.75 ± 0.92[a]	113.380 ± 0.792[a]	1276.0 ± 33.9[a]
	2	2.5800 ± 0.2263[ab]	200.90 ± 1.41[b]	117.415 ± 0.035[b]	1296.0 ± 52.3[a]
	4	2.2000 ± 0.0990[b]	212.20 ± 1.27[c]	119.775 ± 0.474[c]	1386.5 ± 2.1[ab]
	8	1.7050 ± 0.0919[c]	214.30 ± 0.57[c]	123.565 ± 0.389[d]	1436.5 ± 17.7[b]
Tetracycline					
	0	3.5600 ± 0.0141[a]	192.60 ± 5.66[a]	112.605 ± 0.064[a]	1238.5 ± 7.8[a]
	100	3.1850 ± 0.0495[b]	200.65 ± 3.61[ab]	114.010 ± 0.127[b]	1351.5 ± 14.8[b]
	250	2.3700 ± 0.0283[c]	209.80 ± 2.40[bc]	116.835 ± 0.021[c]	1407.5 ± 9.2[bc]
	500	2.3050 ± 0.0071[c]	218.30 ± 0.00[c]	117.645 ± 0.120[d]	1447.0 ± 35.4[c]

±: standard deviation for milk samples containing target antibiotics at selected concentrations

[a-d]values in a column with the same superscript are not significantly different by Fisher's test ($p < 0.05$)

6.6. Fat, Protein, Lactose, Minerals, SNF and Freezing Point Results of Milk Samples

Lactostar is a new developed machine for the routine testing of milk. The measurement is based on a thermo-optical procedure combination. In opto-unit, the undissolved (visible) substances are analyzed such as the sum of fat and protein. It also contains impedance or conductance measurement. In thermal unit, the fat content and the fat-free dry matter are measured.

The fat content of milk and milk products is of significance for the quality. Spectrometric methods are often used to determine fat content. Due to being high cost method, acid butyrometrie is preferred. This method is simple to apply, low-cost. However, there are several drawbacks. It requires practical handling skills (Badertscher, et al. 2007). The Kjeldahl method and dye-binding methods are used for protein determination. The kjeldahl method has long time to analyze and skill required to conduct. Dye-binding method is more rapid method than the Kjeldahl method. Lactose is determined by polarimetric method, gravimetric method, enzymatic method and HPLC method. The ash of milk includes exposing to very high heat. HPLC is preferred for determination of vitamins. HPLC is more complex. The ash content of milk means the total mineral content of milk. For determination of each mineral in milk, atomic absorption spectrophotometry is chosen (Hui 1993). Because of these disadvantages of each method, lactostar usage became a good measurement unit in terms of automatisation such as low-cost, less time.

Milk composition affects on rheological and chemical behavior markedly. Fat, protein, lactose, minerals and solid not fat content of antibiotic free milk samples and milk samples spiked with antibiotics is an important indication of these properties. In addition, increasing false-positive results of several screening test is associated with increasing concentrations of fat, protein, somatic cells, free fatty acids and lactoferrin in milk. For instance, increasing milk protein content caused an increase in false-positive results for the Penzyme screening test (Andrew 2000).

Therefore, this experiment was aimed to investigate the effects of antibiotic concentrations (Penicillin G, Ampicillin, and Tetracycline) on composition of milk samples. The experimental data obtained for milk samples as fat%, protein%, lactose%, SnF%, EMC% and Fpp (°C). By using Minitab 14 trial version, the data were evaluated

statistically. The statistical testing showed that Penicillin G, Ampicillin and Tetracycline concentration had a significant effect on EMC% and conductivity of milk samples at 95% confidence level. In addition, Penicillin G concentration had also a significant effect on Fpp (°C) of milk samples. The freezing point of milk is ranged from -0.53 to 0.59 °C. After high-temperature processing, e.g., ultra heat treatment, sterilization, the freezing point rises because of precipitation of some phosphates (Otter 2003). However, at similar level of the antibiotic concentration, the statistical testing showed no significant difference among the values of fat%, protein%, lactose%, SnF%, at a 95% confidence level. An increase in antibiotic concentration means an increase in EMC% and conductivity but decrease in Fpp (Table 6.13, 6.14. and 6.15).

Table 6.13. Effect of Pencillin G concentration on the Lactostar result of milk samples with using Fisher's test

Penicillin G Concentration (ppb)	Fpp (°C)	EMC% (Minerals)	Conductance (mS)
0	-0.49440 ± 0.00184[a]	0.50533 ± 0.03642[a]	19.615 ± 1.416[a]
2	-0.49573 ± 0.00046[a]	0.53267 ± 0.02219[ab]	20.704 ± 0.747[ab]
4	-0.49107 ± 0.00585[ab]	0.55200 ± 0.02513[bc]	21.370 ± 1.019[bc]
8	-0.48933 ± 0.00690[b]	0.56333 ± 0.03579[c]	21.868 ± 1.367[c]

Table 6.14. Effect of Ampicillin concentration on the Lactostar result of milk samples with using Fisher's test

Ampicillin Concentration (ppb)	EMC%	Conductance (mS)
0	0.50533 ± 0.03642[a]	19.615 ± 1.416[a]
2	0.57667 ± 0.03677[b]	22.299 ± 1.441[b]
4	0.58267 ± 0.04367[b]	22.682 ± 1.641[b]
8	0.59000 ± 0.04472[b]	22.682 ± 1.763[b]

Table 6.15. Effect of Tetracycline concentration on the Lactostar result of milk samples with using Fisher's test

Tetracycline Concentration (ppb)	EMC %	Conductance (mS)
0	0.50533 ± 0.03642^a	19.615 ± 1.416^a
100	0.59467 ± 0.04941^b	23.117 ± 1.791^b
250	0.60000 ± 0.04472^b	23.301 ± 1.796^b
500	0.60600 ± 0.04837^b	23.448 ± 1.855^b

[a-c] values in a column with the same superscript are not significantly different by Fisher's test ($p < 0.05$)

6.7. Confirmation of Target Antibiotics in Milk

The analytical method for the determination of Penicillin G, Ampicillin and Tetracycline residues in milk was conducted. The recovery and precision of the methods were evaluated by analyzing milk samples fortified with Penicillin G at levels of 2, 4 and 8 ppb, Ampicillin at levels of 2, 4 and 8 ppb and Tetracycline at levels of 100, 250 and 500 ppb. Three replicates of samples at each level were analyzed.

6.7.1. Confirmation of Penicillin G in Milk

The retention time of the Penicillin G under UV detection was 5.75 min. Fig. 6.14, Fig. 6.15, Fig. 6.16, and Fig. 6.17 illustrate the chromatograms of milk sample blank, milk samples fortified with Penicillin G (2, 4, 8 ppb) at 325 nm, respectively. The chromatogram of blank milk sample showed no peak interfering with Penicillin G.
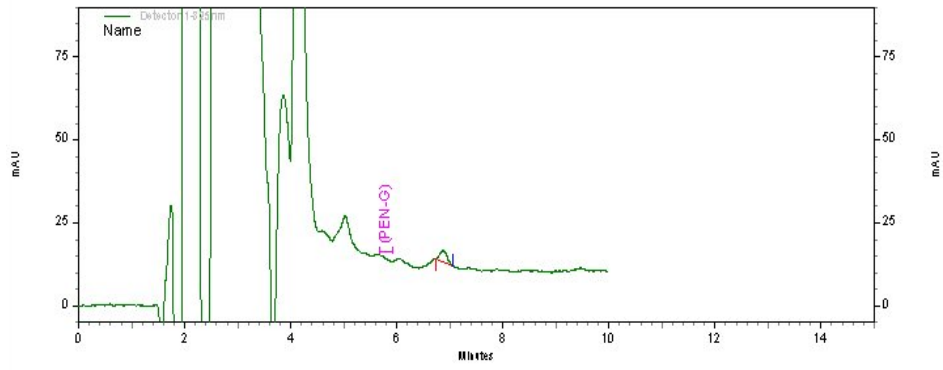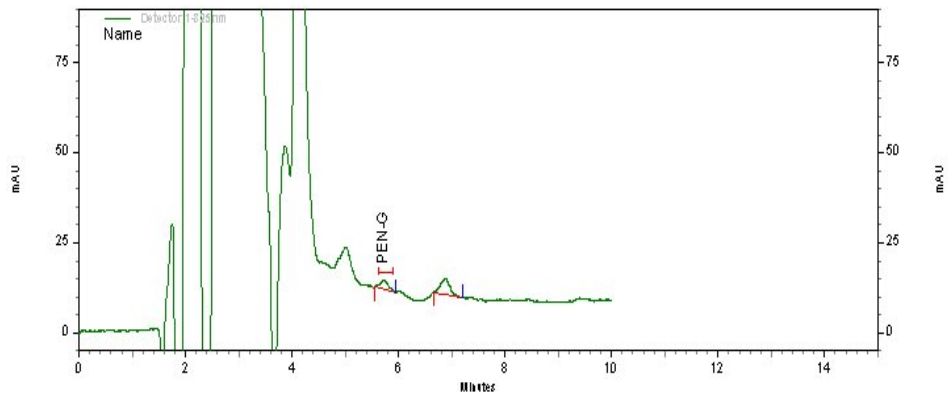
Name	Time	Area	Amount	Height	Units	Start Time	Stop Time
PEN-G			0.0 BDL		PPB		
Totals							

Figure 6.14. Chromatograms of milk sample blank for Penicillin G

Name	Time	Area	Amount	Height	Units	Start Time	Stop Time
PEN-G	5.73	28960	11.8	2632	PPB	5.55	5.96
Totals		28960	11.8	2632			

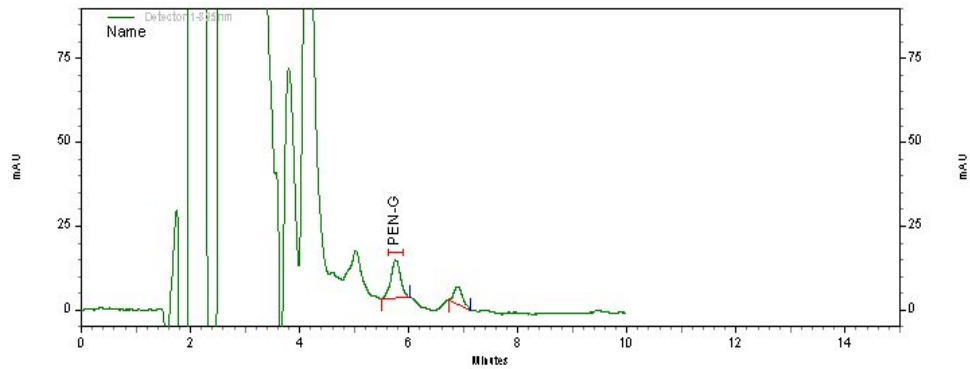Figure 6.15. Chromatograms of milk sample fortified with Penicillin G at 2 ppb
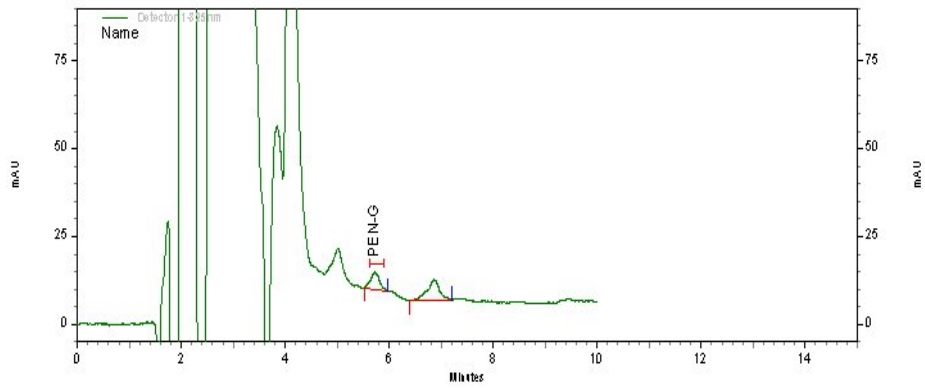
Detector 1-325nm Results (System
(6/25/2008 5:05:24 PM)
(Reprocessed))

Name	Time	Area	Amount	Height	Units	Start Time	Stop Time
PEN-G	5.72	60046	24.4	5032	PPB	5.51	5.97
Totals		60046	24.4	5032			

Figure 6.16. Chromatograms of milk sample fortified with Penicillin G at 4 ppb

Detector 1-325nm Results (System
(6/25/2008 4:21:41 PM)
(Reprocessed))

Name	Time	Area	Amount	Height	Units	Start Time	Stop Time
PEN-G	5.76	137384	55.9	11201	PPB	5.51	6.02
Totals		137384	55.9	11201			

Figure 6.17. Chromatograms of milk sample fortified with Penicillin G at 8 ppb

The linearity of the calibration curve for Penicillin G was good (R^2= 0.997166) for a range of concentrations 40, 80, 160, 320 ppb of Penicillin G in the milk samples (Appendix C). The linear regression equation was y=ax+b; (a) 2461.89, (b) 0.000000.

Milk samples were spiked with Penicillin G at 2, 4, 8 ppb. Three replicates of milk samples at each level were analyzed. Recoveries are shown in Table 6.16. The recoveries were obtained by spiking milk samples at three different concentrations (2, 4, 8 ppb). Recoveries ranged from 44.667 to 66.000%.

Table 6.16. Recoveries of penicillin G from fortified milk samples

Spiked (ppb)	Recovery %
2	44.667 ± 12.662
4	59.333 ± 1.528
8	66.000 ± 3.606

6.7.2. Confirmation of Ampicillin in Milk

Several experiments were made to develop an HPLC method for determination of ampicillin in ppb level concentrations in milk samples. HPLC with fluorescence detection was prefered. The method of Gamba and Dusi (2003) was followed for the determination of ampicillin residues in milk samples. A HPLC chromatogram of ampicillin residues at 0, 2, 4, 8 ppb concentrations in UHT cow's milk is demonstrated in Figure 6.18, 6.19, 6.20 and 6.21. The retention time for milk containing ampicillin at 0, 2, 4, 8 ppb levels were 7.75 min. Blank milk had no interfering peaks.
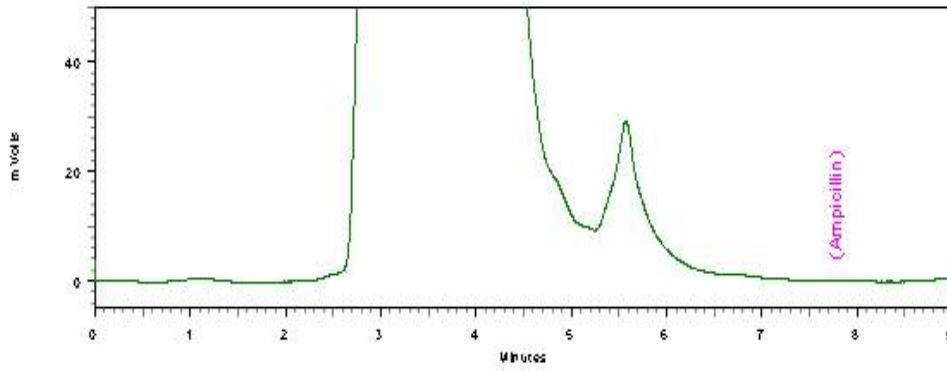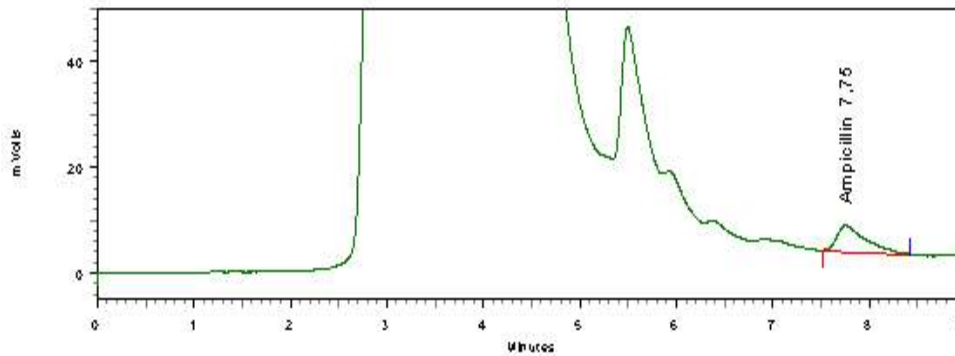
Retention Time	Name	Area	ESTD concentration
	Ampicillin		0,0 BDL
Totals			

Figure 6.18. Chromatograms of milk sample blank for Ampicillin

Retention Time	Name	Area	ESTD concentration
7,75	Ampicillin	101312	1,1
Totals		101312	1,1

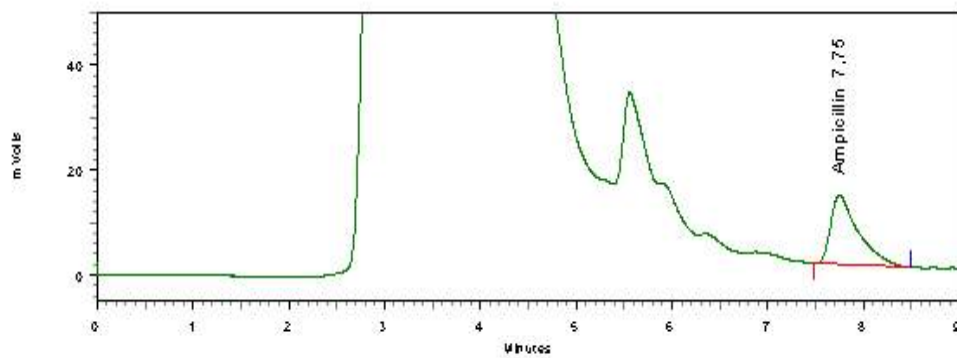Figure 6.19. Chromatograms of milk sample fortified with Ampicillin at 2 ppb
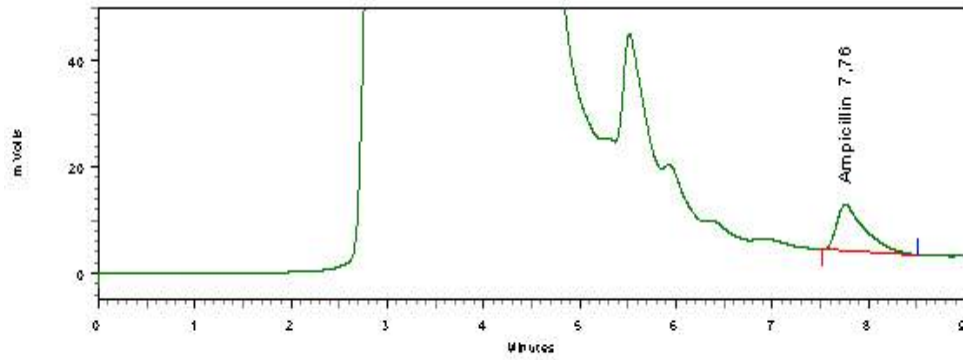
Retention Time	Name	Area	ESTD concentration
7,76	Ampicillin	178008	1,9
Totals		178008	1,9

Figure 6.20. Chromatograms of milk sample fortified with Ampicillin at 4 ppb

Retention Time	Name	Area	ESTD concentration
7,75	Ampicillin	268322	2,9
Totals		268322	2,9

Figure 6.21. Chromatograms of milk sample fortified with Ampicillin at 8 ppb

Quantification was performed by comparison of the analyte peak areas versus an externally generated calibration curve. Calibration curve was produced at detection the excitation wavelenght of 346 nm and the emission wavelenght of 425 nm for the ampicillin in the concentration range of 2-16 ppb (Appendix C). The calibration curve was linear over the the concentration range tested with goodness of fit (R^2) 0.998702. Its linear fit was y=ax+b; (a) 1.06572e-005, (b) 0.

The recoveries of ampicillin from milk samples spiked at concentrations of 2, 4, 8 ppb were determined. The mean percentage recoveries with standard deviation were presented in Table 6.17.

Table 6.17. Recoveries of ampicillin from fortified milk samples

Spiked (ppb)	Recovery %
2	87.520 ± 8.660
4	73.740 ± 4.330
8	62.503 ± 10.984

6.7.3. Confirmation of Tetracycline in Milk

Typical chromatogram of tetracycline standard at 250, 500, 750, 1000 ppb monitored at 360 nm were shown in Figure C.6, C.7, C.8 and C.9 (Appendix C). The method of Cinquina, et al. (2003) was followed for the determination of tetracycline residues in milk samples. The chromatograms of blank milk sample and of milk samples spiked with 100, 250 and 500 ppb of tetracycline were reported in Figure 6.22, 6.23, 6.24 and 6.25. No interference was observed in the blank milk sample chromatograms (Figure 6.22). The retention time of milk samples containing tetracycline was 4.51 min.

The linearity of the calibration curve for tetracycline was good (R^2=0.995229) for 250, 500, 750, 1000 ppb of tetracycline in the milk samples. The linear regression equation was y=ax+b. The coefficients (a and b) were 0.000609639 and 0.00000, respectively.
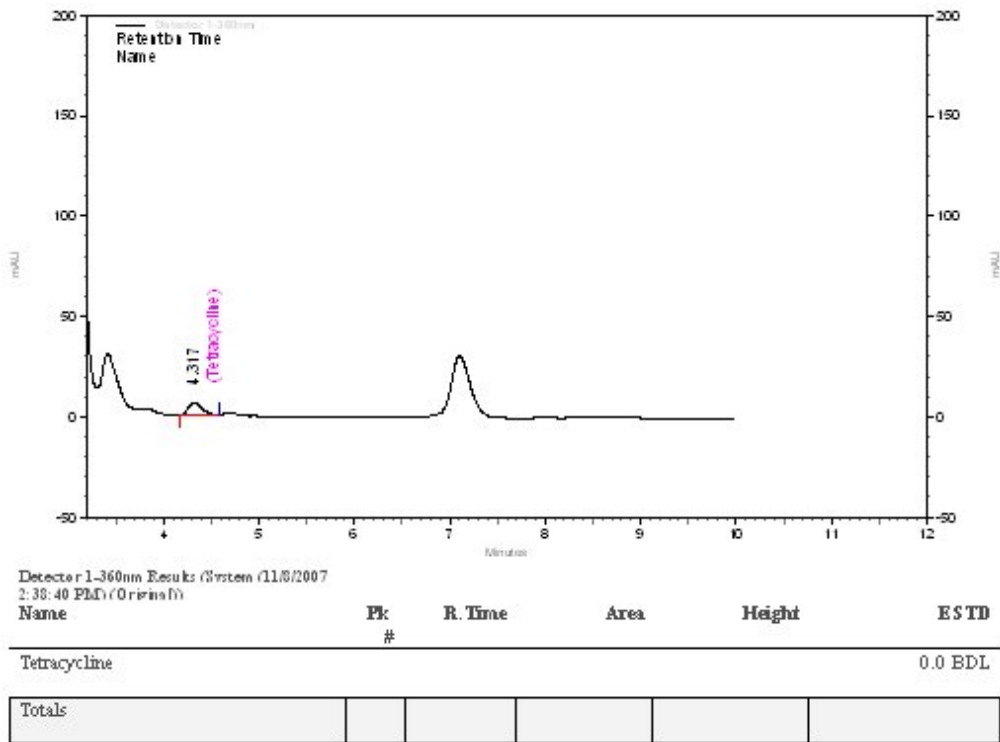
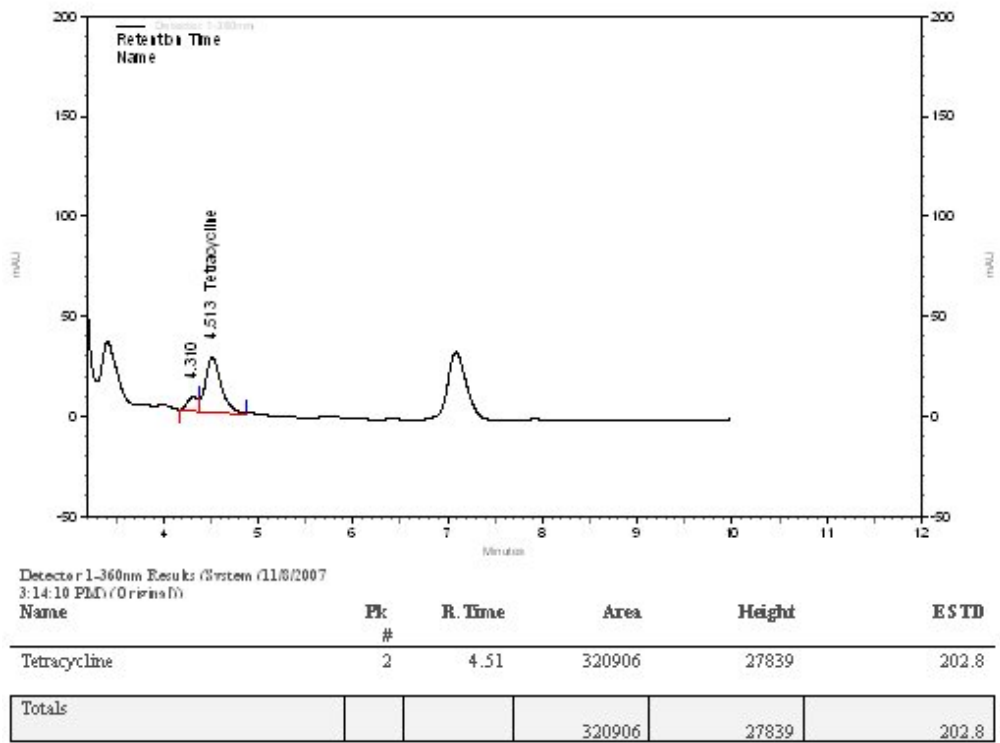Figure 6.22. Chromatograms of milk sample blank for Tetracycline

Figure 6.23. Chromatograms of milk samples fortified with Tetracycline at 100 ppb
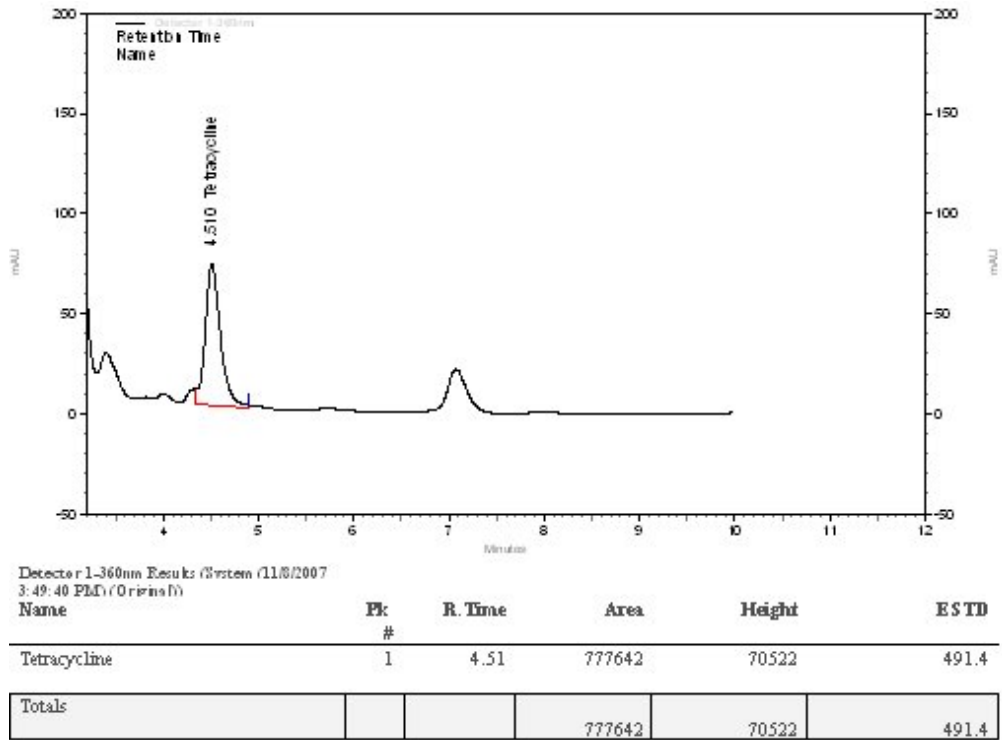
Detector 1-360nm Results (System (11/8/2007 3:49:40 PM) (Original) Name	Pk #	R. Time	Area	Height	ESTD
Tetracycline	1	4.51	777642	70522	491.4
Totals			777642	70522	491.4

Figure 6.24. Chromatograms of milk samples fortified with Tetracycline at 250 ppb

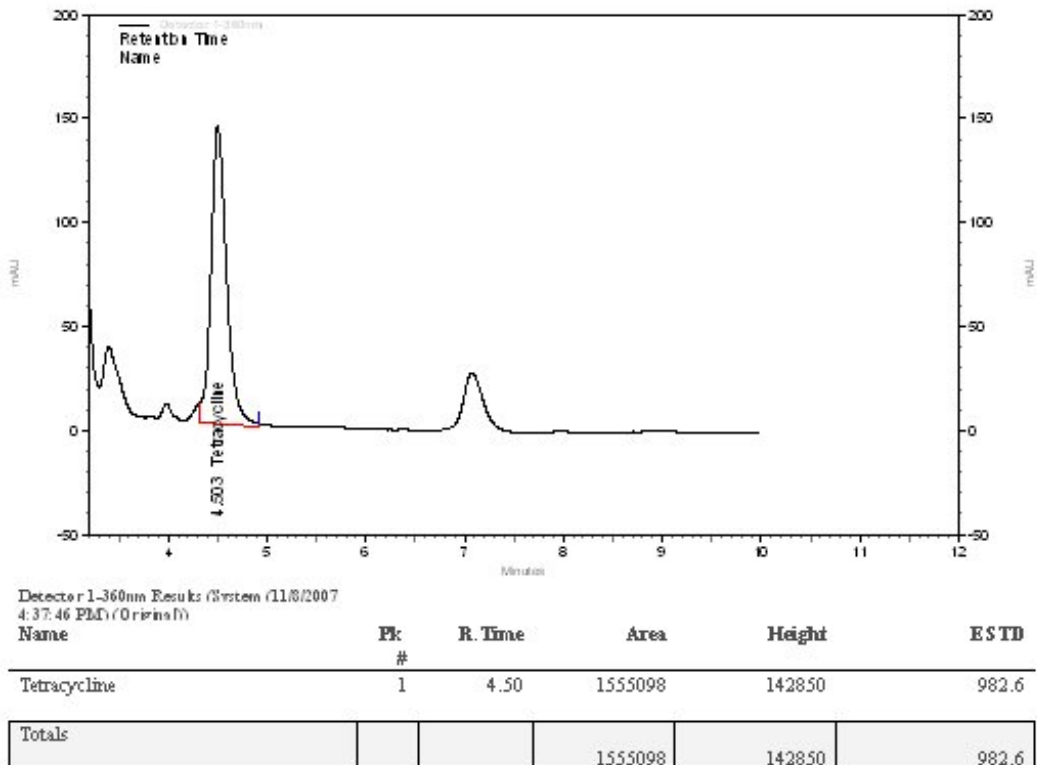| Detector 1-360nm Results (System (11/8/2007 4:37:46 PM) (Original) Name	Pk #	R. Time	Area	Height	ESTD
Tetracycline	1	4.50	1555098	142850	982.6
Totals			1555098	142850	982.6

Figure 6.25. Chromatograms of milk samples fortified with Tetracycline at 500 ppb

Milk samples were fortified with tetracycline at 100, 250 and 500 ppb levels. Three replicates of milk samples at each level were analyzed. Recoveries are shown in Table 6.18. The recoveries were obtained by spiking milk samples at three different concentrations (100, 250, 500 ppb).

Table 6.18. Recoveries of tetracycline from fortified milk samples

Spiked (ppb)	Recovery %
100	93.10 ± 18.04
250	94.35 ± 4.29
500	92.86 ± 5.87

CHAPTER 7

CONCLUSION

There are several screening and confirmatory methods for detecting antibiotic residues in milk and milk products. The major interest in future trend is development of more sensitive, more rapid, simple, specific and easily automatable test.

In this study, firstly different test kits such as Copan Milk Test, Penzyme and ROSA Test were evaluated according to usage, cost and storage time. Comparison of the tests, Copan Milk Test was easy to use, easy to interperate visually and had a long shelf life. The ROSA test is a good immuno-receptor test because it is fast to run, simple to use but needs to have a ROSA Reader device and different strips for different antibiotics. Penzyme Milk Test had very short shelf life and the test is based on having a rather narrow antibiotic spectrum including only beta-lactams as compared to two other tests.

These screening milk tests were confirmed by HPLC for Penicillin G, Ampicillin and Tetracycline residues in UHT cow's milk samples, by using analytical confirmatory method such as a HPLC method. Average recoveries for Ampicillin and Tetracycline ranged, respectively, from 44.67% to 66.00%, from 62.50% to 87.52% and from 92.86% to 94.35%.

Secondly, the effect of antibiotic concentration (Penicillin G, Ampicillin, Tetracycline) on some physical properties (acidity, pH and density) of UHT whole cow's milk (at least 3.1% fat) were examined as well as EC of milk was measured using conductivity meter. The acidity, pH and density of milk were found to be independent of antibiotic concentration level.

Experimental data of EC were evaluated by applying the statistical methods (ANOVA with Fisher's test and PNN method). Based on the results, EC of milk seems to be proper detector for antibiotic residues. This evaluation indicates that EC measurement can be a great promising technique as a rapid, inexpensive, easy-use tool for detection of antibiotic residues in milk. Further development can include developing a computer program that will increase the speed, sensitivity, accuracy of measurement and calculation. On-line EC data may be combined with other significant on-line data,

such as milk composition. These data can be used to detect the any milk samples containing antibiotic in the EC database of dairy farms. In addition, it can be a step in order to develop a sensor for antibiotic residue detection of milk.

The thermal behavior of antibiotic free milk samples and milk samples fortified with antibiotics can be characterized by transition temperatures in DSC curves. An increase in antibiotic concentration resulted in an increase in values of thermal properties, i.e., melting temperature, heat of fusion, evaporation temperature and heat of evaporation of milk samples. It can be concluded that DSC is a good characterization tool for better understanding of thermal events in antibiotic free milk samples and milk samples spiked with antibiotic residues and it can be evaluated as a detection method.

Milk is a very complex because of having different components, such as lipids, proteins, vitamins, minerals and also somatic cells and bacteria. This composition of milk may have a significant effect on the detection of antibiotic residues in milk. Furthermore, it could be demonstrated by Lactostar measurements that the presence of antibiotic residues in milk influenced freezing point (Fpp, °C) and EMC%.

Thus, this study can be subject to further research to evaluate its broad application in other antibiotic residues to detect in milk matrix. However, the further research needs to be confirmed with naturally contaminated milk samples from treated cows with other significant antibiotic residues. Moreover, there is not any efficient antibiotic residue database and antibiotic residue prevention program in Turkey. It is considered that such programs may have benefits for reducing the risk of antibiotic residue violations. Therefore, this work may lead further studies in this area.

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APPENDICES
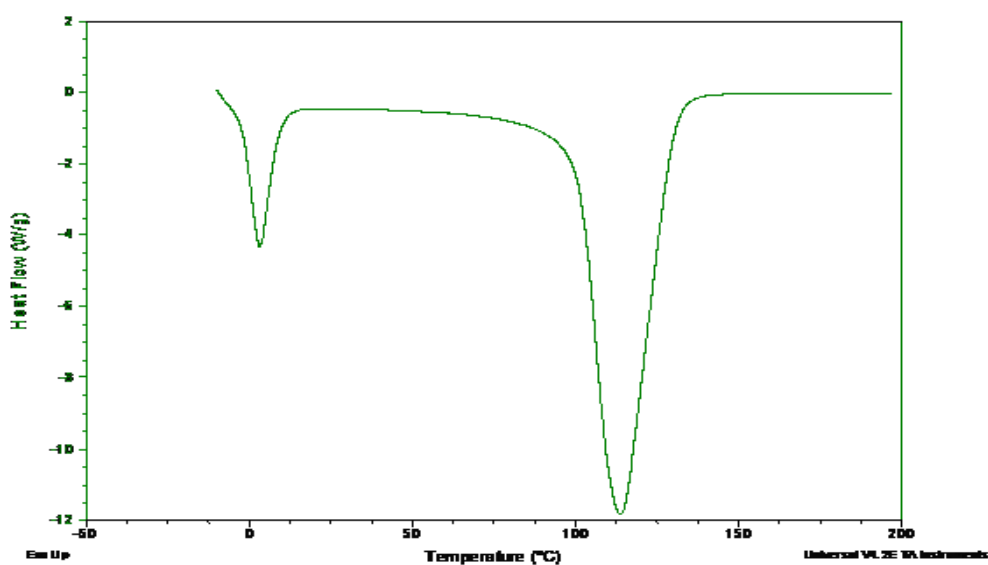
APPENDIX A

DSC RESULTS OF PENICILLIN G RESIDUES
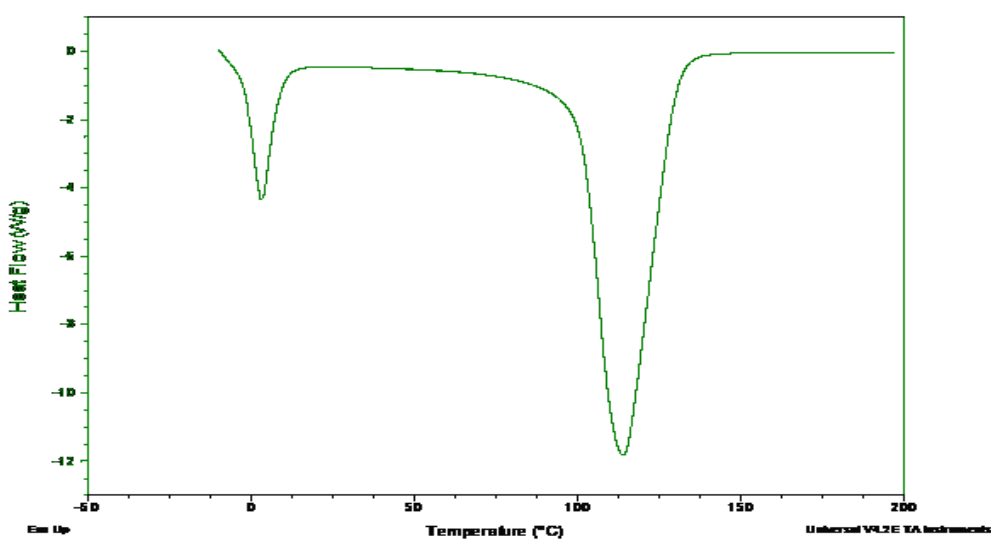

Figure A.1. DSC results of antibiotic free milk sample

Figure A.2. DSC results of milk sample containing 2 ppb Penicillin G
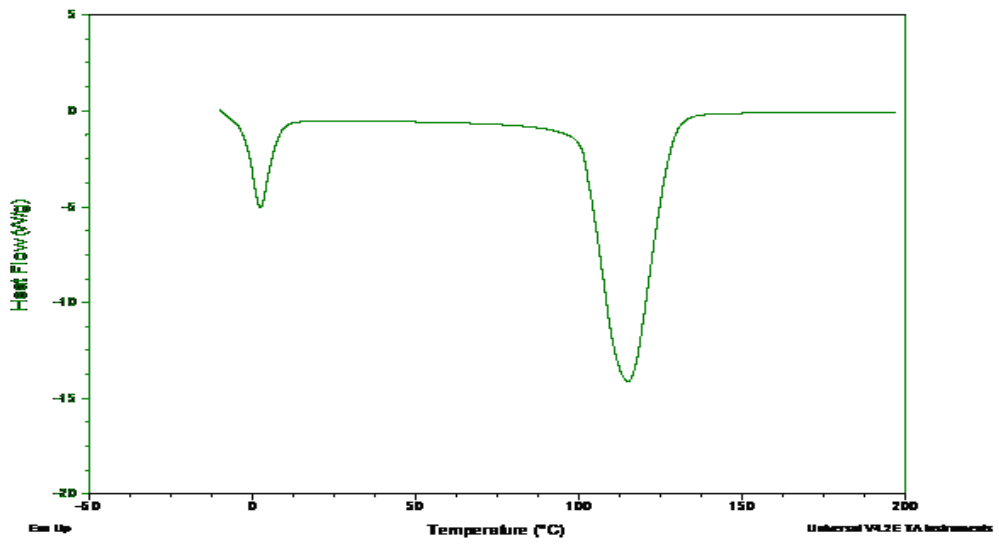
Figure A.3. DSC results of milk sample containing 4 ppb Penicillin G
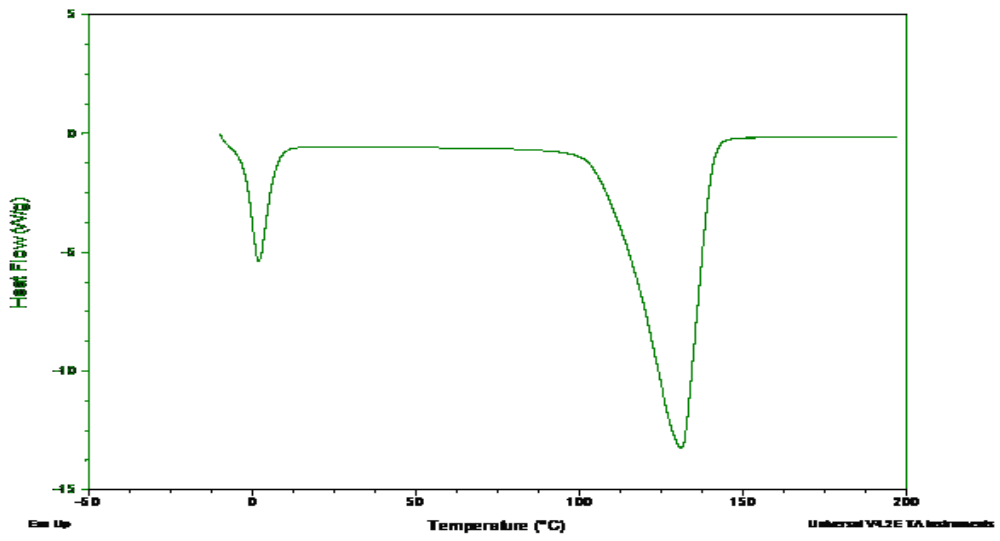
Figure A.4. DSC results of milk sample containing 8 ppb Penicillin G

DSC RESULTS OF AMPICILLIN RESIDUES
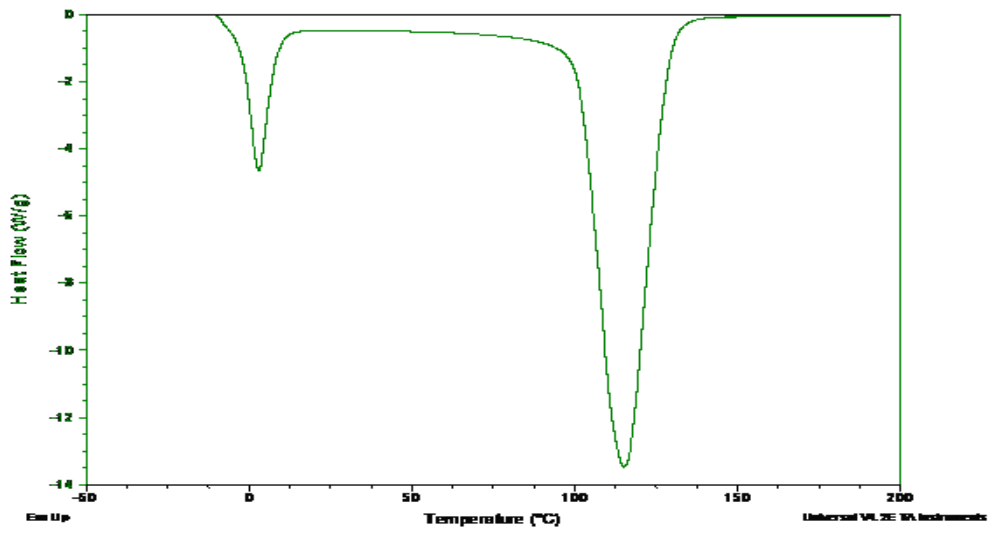
Figure A.5. DSC results of milk sample containing 2 ppb Ampicillin
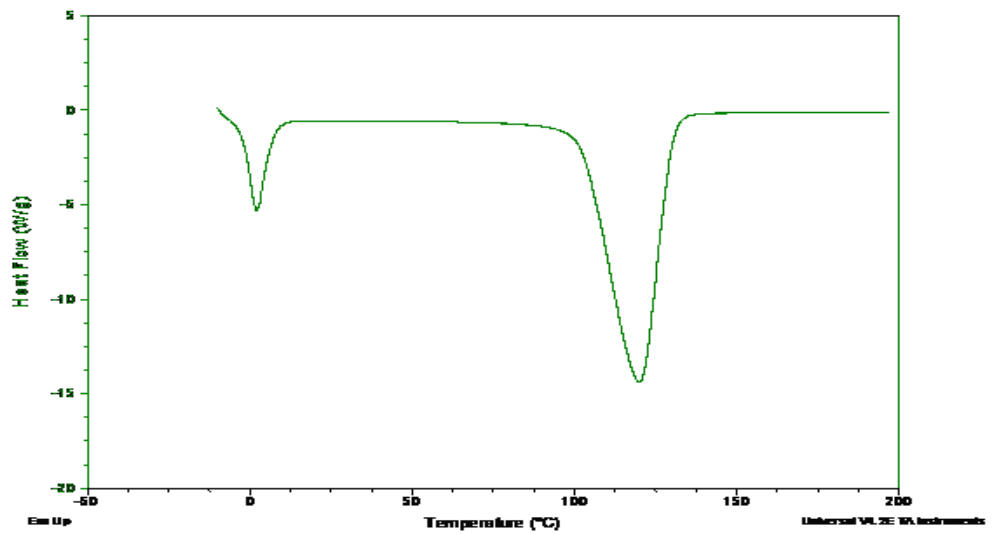
Figure A.6. DSC results of milk sample containing 4 ppb Ampicillin
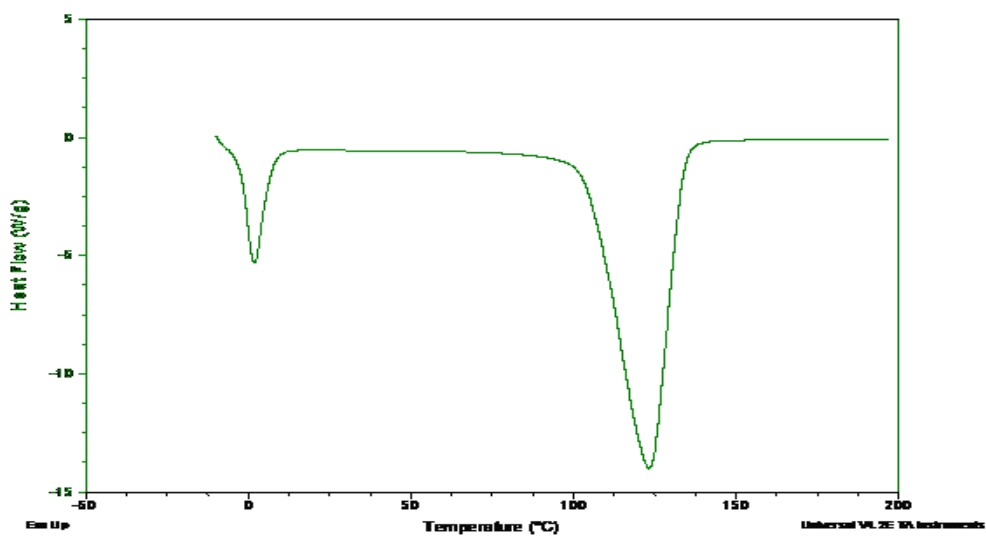
Figure A.7. DSC results of milk sample containing 8 ppb Ampicillin

DSC RESULTS OF TETRACYCLINE RESIDUES
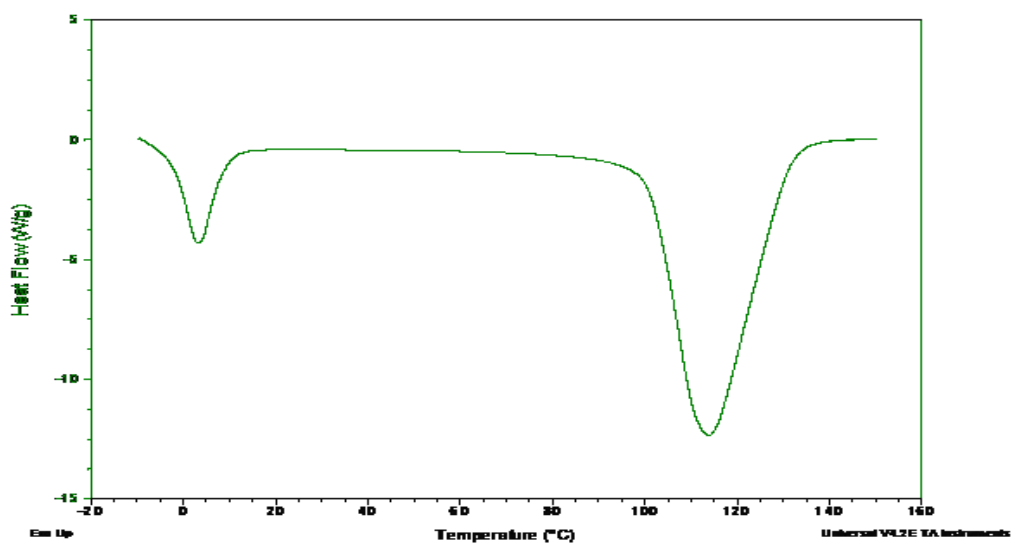
Figure A.8. DSC results of milk sample containing 100 ppb Tetracycline
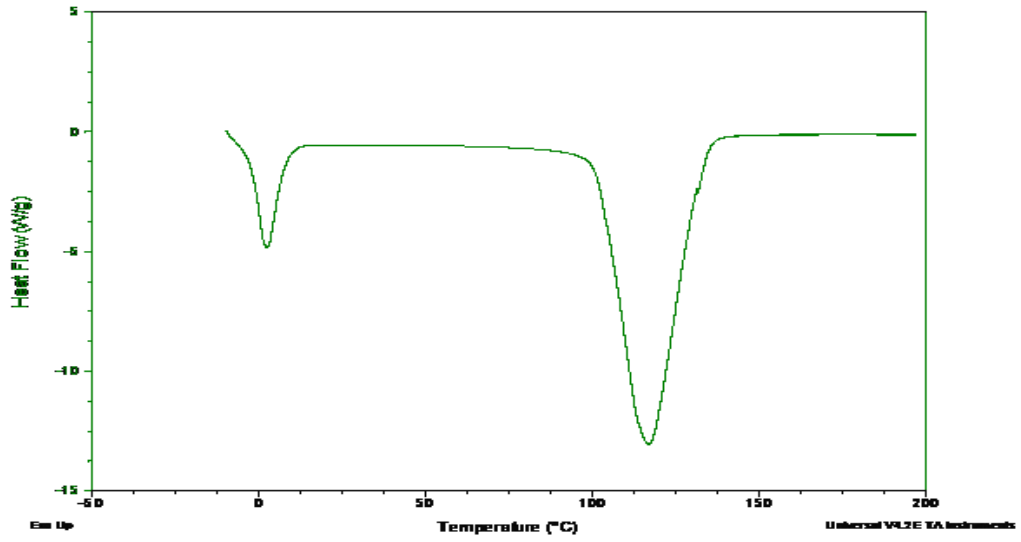
Figure A.9. DSC results of milk sample containing 250 ppb Tetracycline
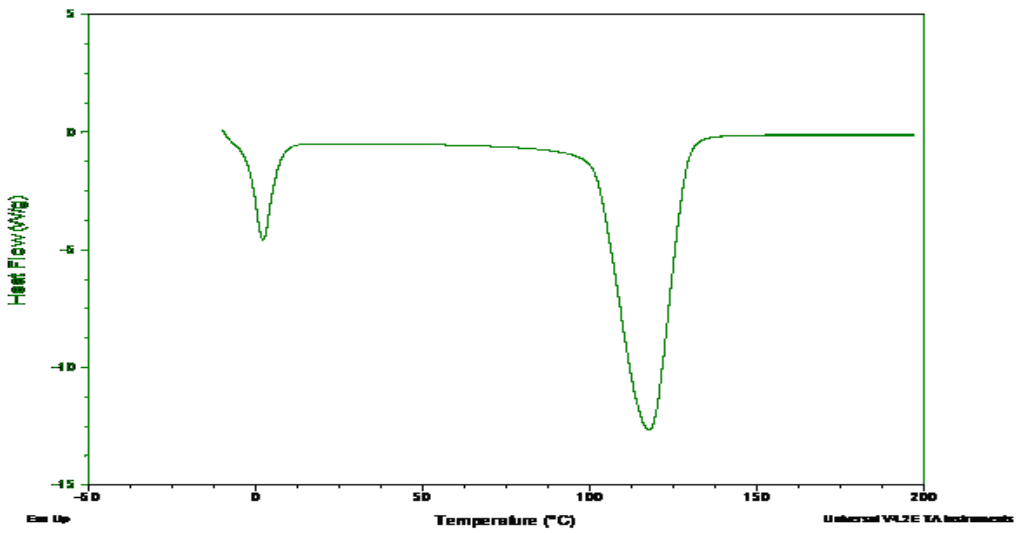
Figure A.10. DSC results of milk sample containing 500 ppb Tetracycline

APPENDIX B

Table B.1. Insignificant Lactostar results

Antibiotic Concentration (ppb)	Fat %	Protein %	Lactose %
Penicillin G			
0 ppb	3.2193 ± 0.1207	3.2447 ± 0.0155	4.7147 ± 0.0229
2 ppb	3.2227 ± 0.1166	3.2327 ± 0.0291	4.6967 ± 0.0412
4 ppb	3.1513 ± 0.1770	3.2320 ± 0.0300	4.6993 ± 0.0388
8 ppb	3.1673 ± 0.1230	3.2193 ± 0.0383	4.6827 ± 0.0512
Ampicillin			
0 ppb	3.2193 ± 0.1207	3.2447 ± 0.0155	4.7147 ± 0.0229
2 ppb	3.1393 ± 0.1825	3.2220 ± 0.0384	4.6860 ± 0.0507
4 ppb	3.1667 ± 0.1387	3.2153 ± 0.0494	4.6767 ± 0.0666
8 ppb	3.1387 ± 0.1927	3.2187 ± 0.0449	4.6800 ± 0.0602
Tetracycline			
0 ppb	3.2193 ± 0.1207	3.2447 ± 0.0155	4.1747 ± 0.0229
100 ppb	3.1680 ± 0.1323	3.2160 ± 0.0484	4.6773 ± 0.0656
250 ppb	3.1247 ± 0.2004	3.2220 ± 0.0446	4.6873 ± 0.0570
500 ppb	3.1767 ± 0.1356	3.2180 ± 0.0490	4.6807 ± 0.0653
Used milk at experiment (at least 3.1% fat, 100ml)	3.1 g	3.1 g	4.7 g
Literature (Fox and McSweeney 1998)	3.4 - 5.1 % [w/v]	3.3 - 3.9 % [w/v]	4.9 - 5.1 % [w/v]

APPENDIX C

CALIBRATION CURVES FOR PENICILLIN G
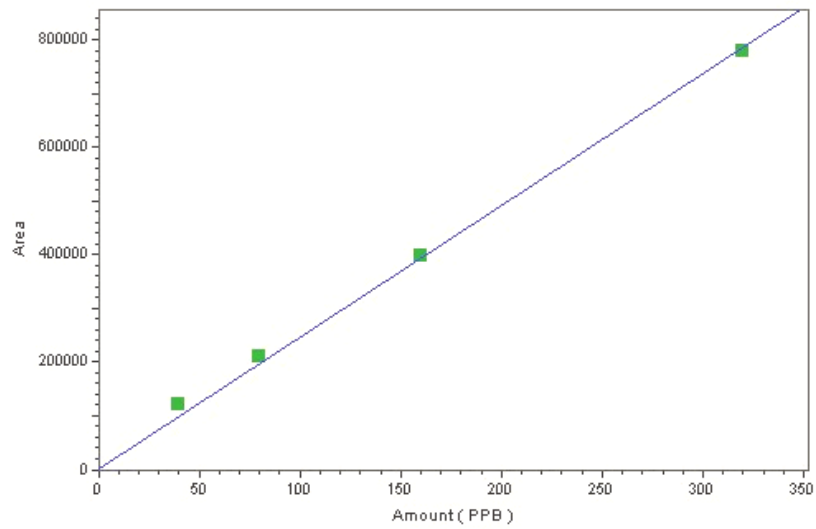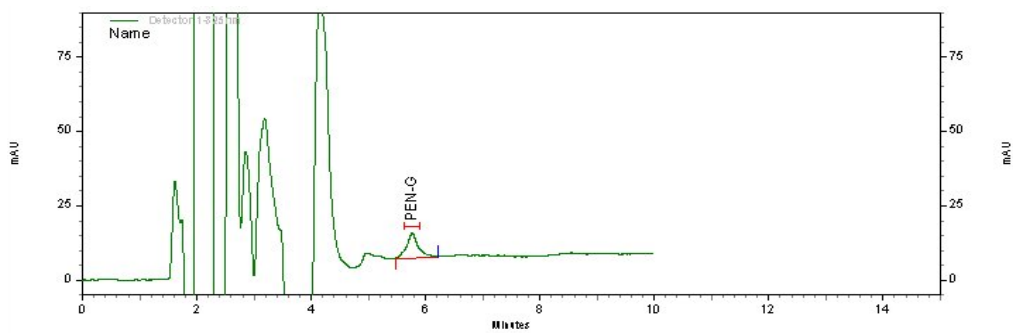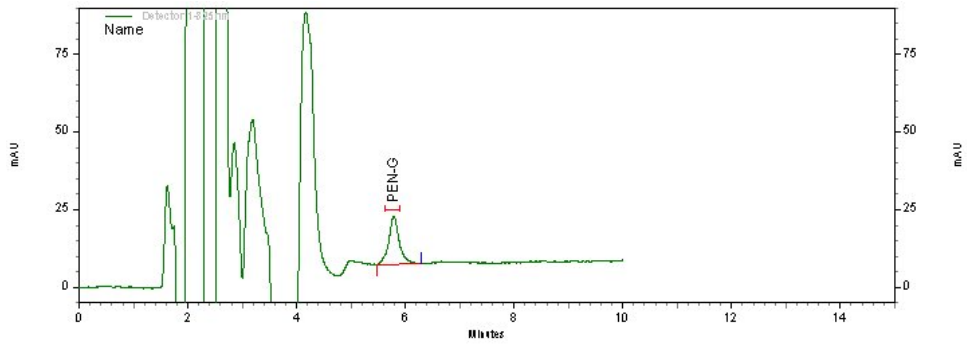
AMPICILLIN AND TETRACYCLINE

Figure C.1. Calibration plot for Ampicillin for concentration range of 2 ppb-16 ppb

Detector 1-325nm Results (System
(6/25/2008 4:19:49 PM)
(Reprocessed))

Name	Time	Area	Amount	Height	Units	Start Time	Stop Time
PEN-G	5.76	115031	40.0 CAL	8106	PPB	5.47	6.21
Totals		115031	40.0 CAL	8106			

Figure C.2. Plot for 40 ppb Penicillin G standard

Name	Time	Area	Amount	Height	Units	Start Time	Stop Time
PEN-G	5.79	207299	80.0 CAL	15295	PPB	5.48	6.29
Totals		207299	80.0 CAL	15295			

Figure C.3. Plot for 80 ppb Penicillin G standard
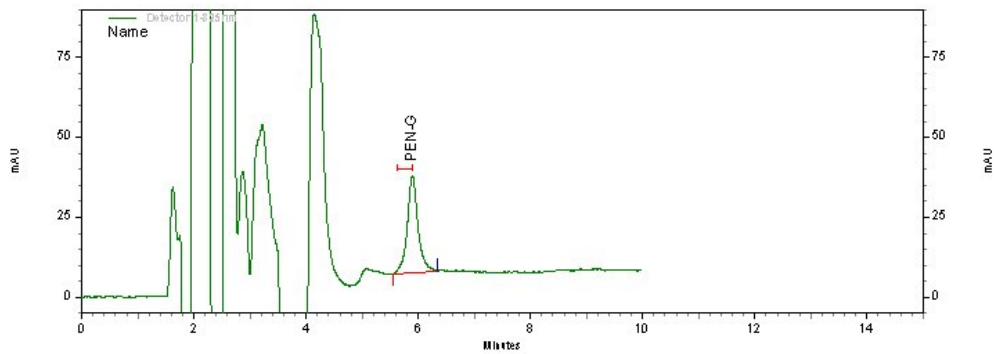
Name	Time	Area	Amount	Height	Units	Start Time	Stop Time
PEN-G	5.89	398145	160.0 CAL	30175	PPB	5.56	6.34
Totals		398145	160.0 CAL	30175			

Figure C.4 Plot for 160 ppb Penicillin G standard
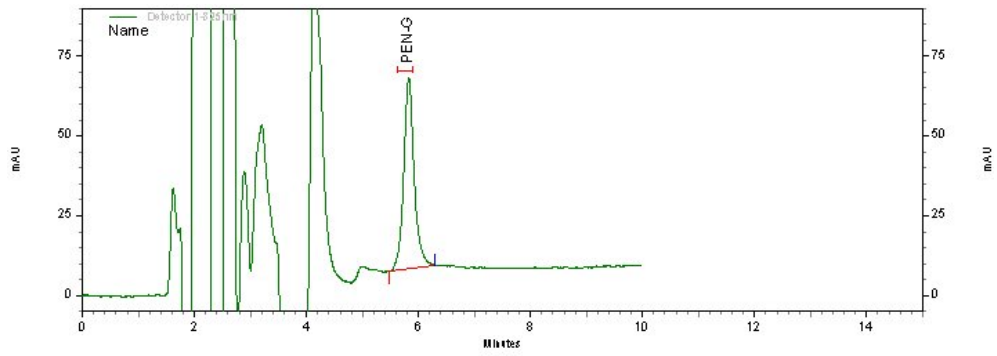
116

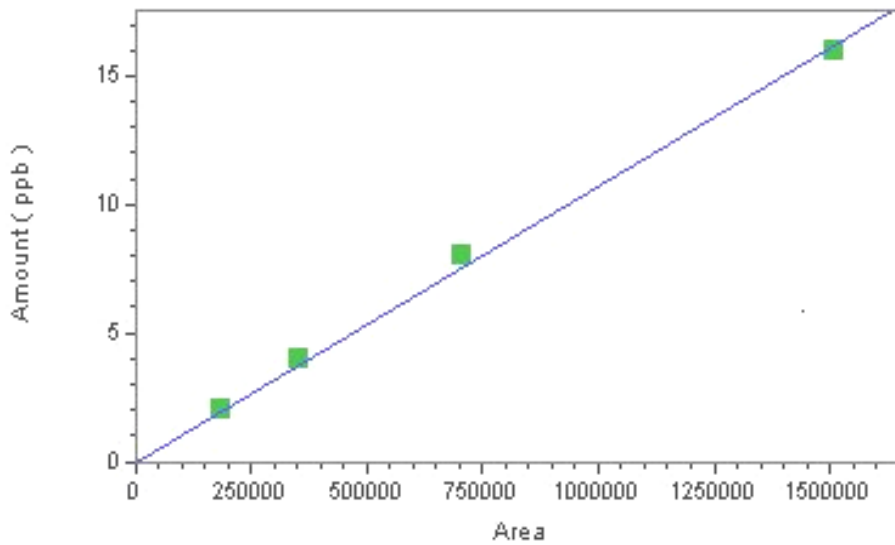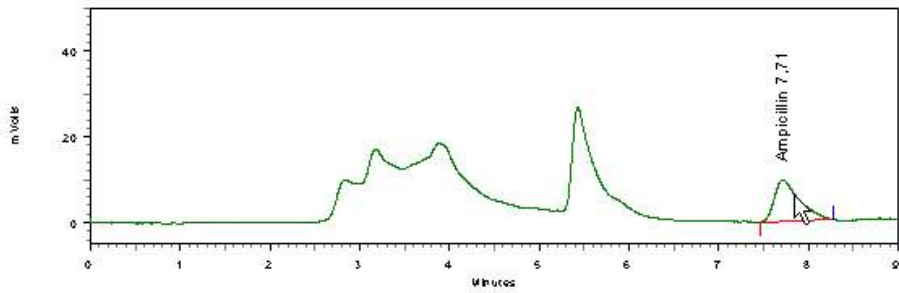| Name	Time	Area	Amount	Height	Units	Start Time	Stop Time
PEN-G	5.83	779654	320.0 CAL	59886	PPB	5.48	6.30
Totals		779654	320.0 CAL	59886			

Figure C.5. Plot for 320 ppb Penicillin G standard

Figure C.6. Calibration plot for Ampicillin for concentration range of 2 ppb-16 ppb

SAMPLE CODE: std2ppb
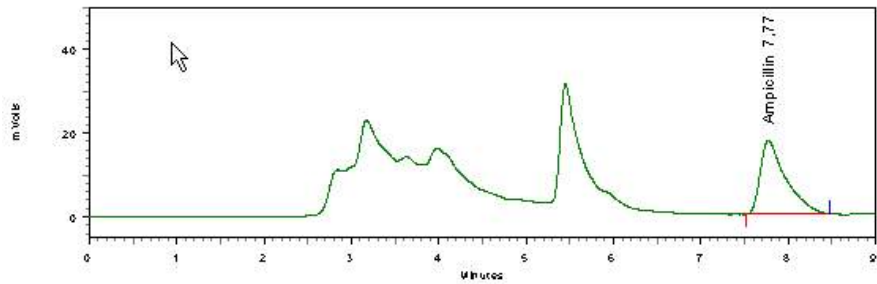

Detector A (Ex:346nm,
Em:425nm)

Retention Time	Name	Area	ESTD concentration
7,71	Ampicillin	185527	2,0 CAL
Totals		185527	2,0 CAL

Figure C.7. Plot for 2 ppb Ampicillin standard

SAMPLE CODE: std4ppb

Detector A (Ex:346nm,
Em:425nm)

Retention Time	Name	Area	ESTD concentration
7,77	Ampicillin	356139	4,0 CAL
Totals		356139	4,0 CAL

Figure C.8. Plot for 4 ppb Ampicillin standard

SAMPLE CODE: STD 8 PPB
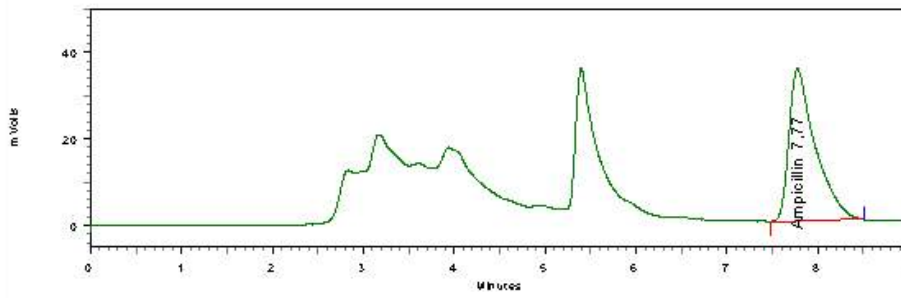
Detector A (Ex:346nm,
Em:425nm)

Retention Time	Name	Area	ESTD concentration
7,77	Ampicillin	705988	7,6
Totals		705988	7,6

Figure C.9. Plot for 8 ppb Ampicillin standard

SAMPLE CODE: std16ppb
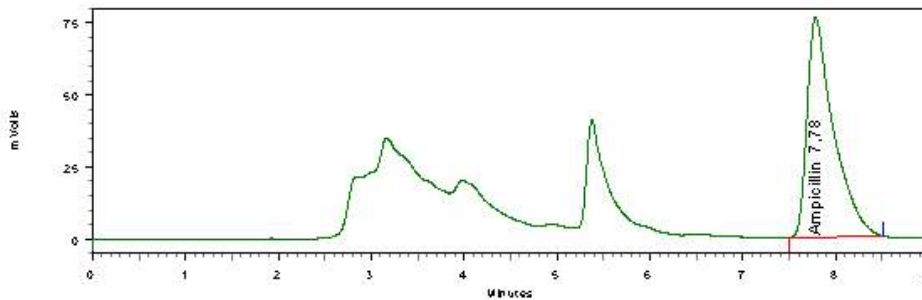
Detector A (Ex:346nm,
Em:425nm)

Retention Time	Name	Area	ESTD concentration
7,78	Ampicillin	1508641	16,0 CAL
Totals		1508641	16,0 CAL

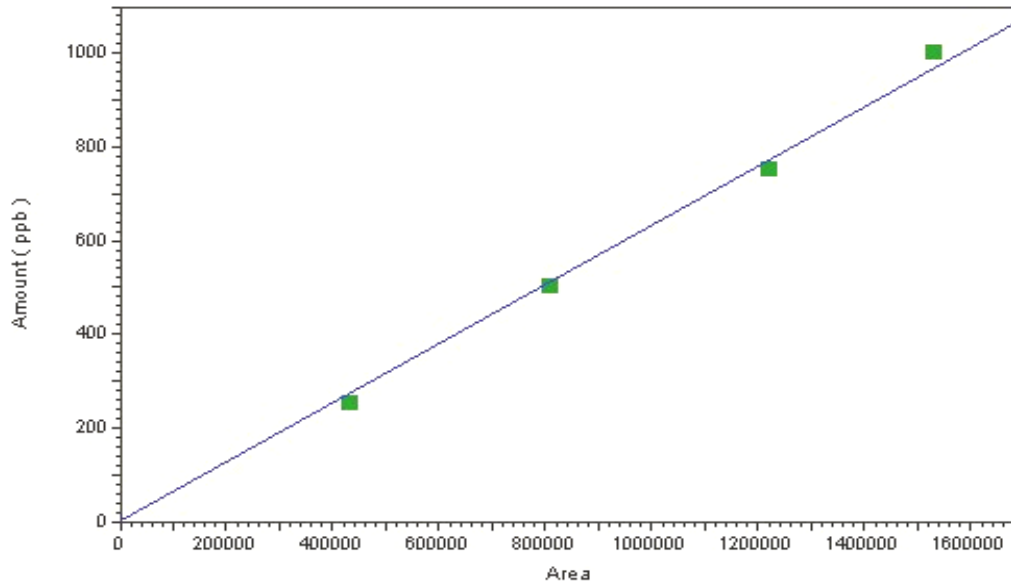Figure C.10. Plot for 16 ppb Ampicillin standard

119

Figure C.11. Calibration plot for Tetracycline for concentration range of 250 ppb-1000 ppb
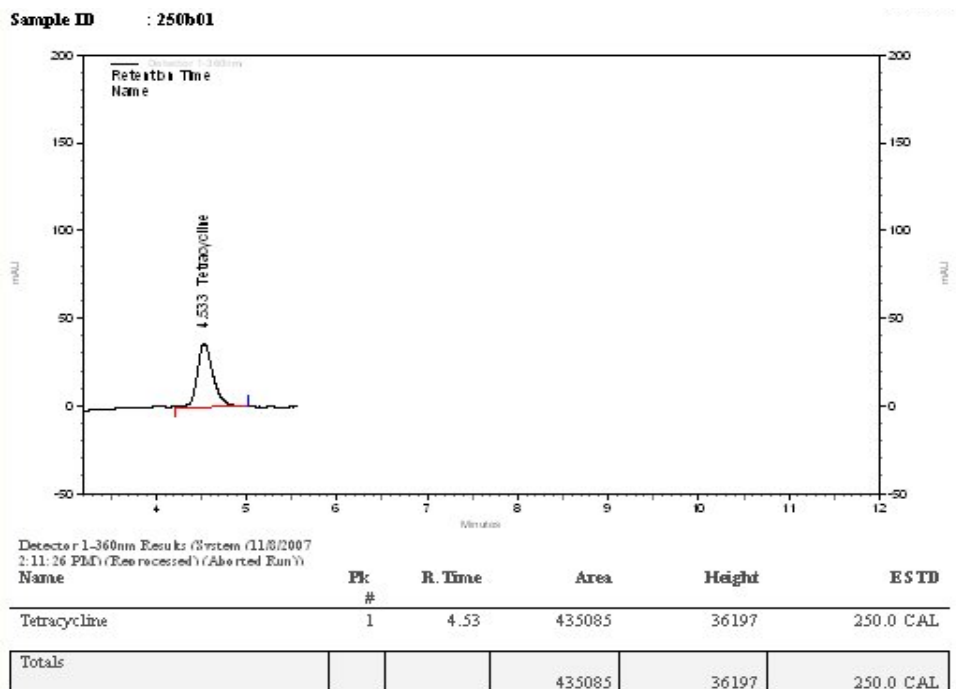

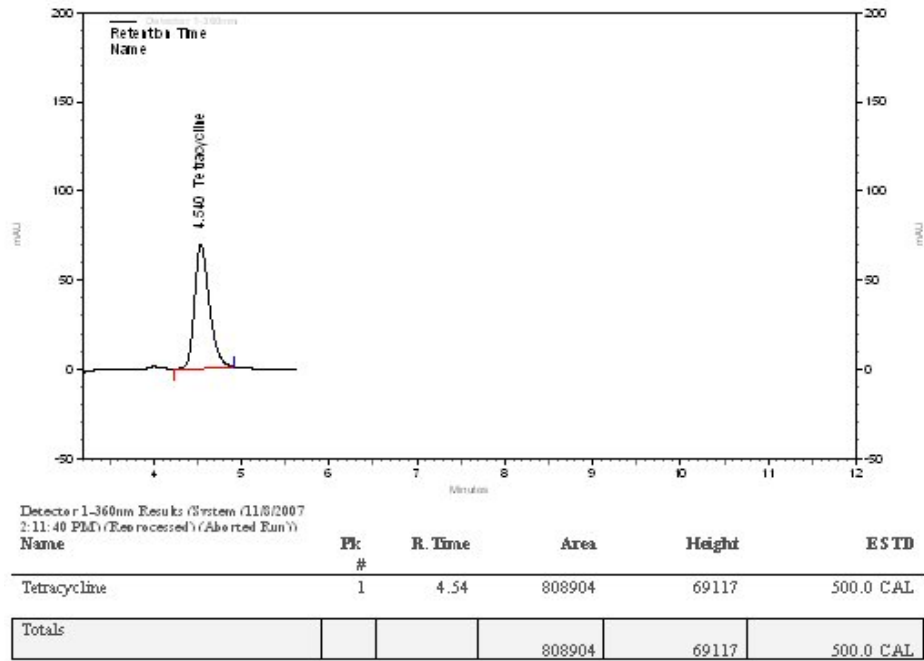Figure C.12. Plot for 250 ppb Tetracycline standard

Name	Pk #	R. Time	Area	Height	ESTD
Tetracycline	1	4.54	808904	69117	500.0 CAL
Totals			808904	69117	500.0 CAL

Detector 1–360nm Results (System (11/8/2007 2:11:40 PM) (Reprocessed) (Aborted Run))

Figure C.13. Plot for 500 ppb Tetracycline standard

Name	Pk #	R. Time	Area	Height	ESTD
Tetracycline	1	4.54	1223377	102436	750.0 CAL
Totals			1223377	102436	750.0 CAL

Detector 1–360nm Results (System (11/8/2007 2:11:54 PM) (Reprocessed) (Aborted Run))
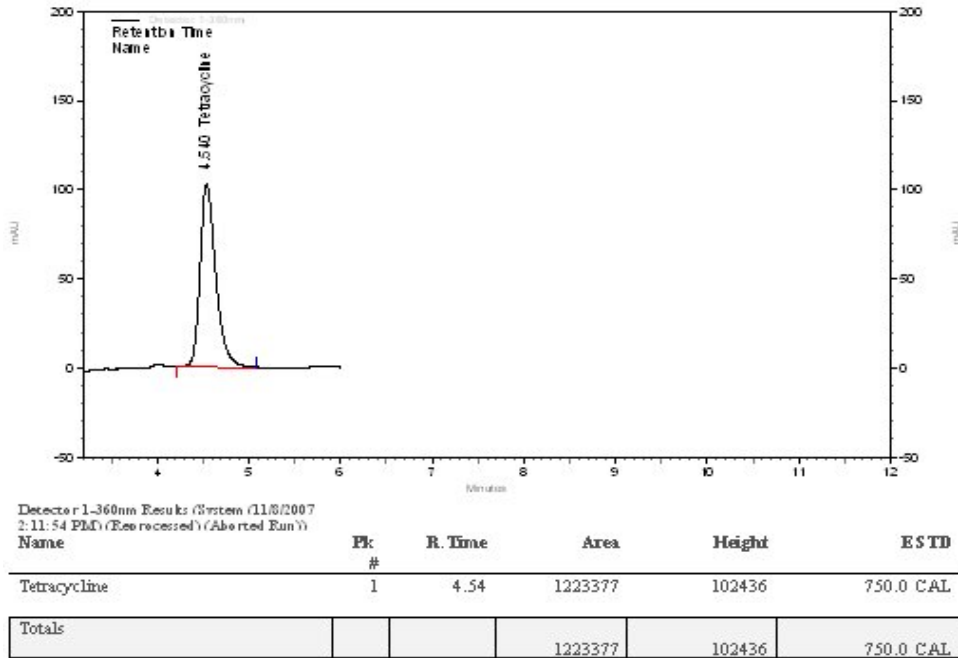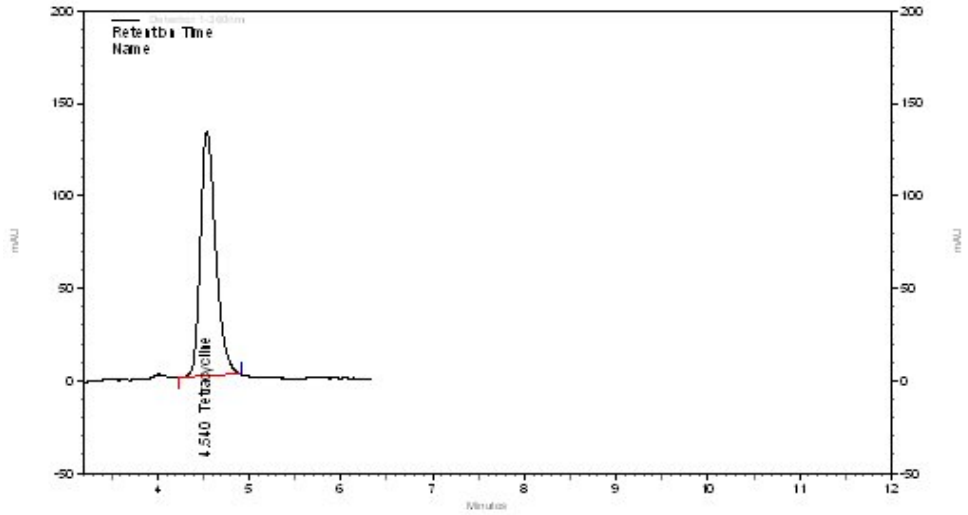
Figure C.14. Plot for 750 ppb Tetracycline standard

121

Figure C.15. Plot for 1000 ppb Tetracycline standard

Detector 1-360nm Results (System (11/8/2007
2:12:09 PM) (Reprocessed) (Aborted Run))

Name	Pk #	R. Time	Area	Height	ESTD
Tetracycline	1	4.54	1533174	132061	1000.0 CAL
Totals			1533174	132061	1000.0 CAL