THE CONFIRMATION OF THE COMMERCIAL KITS USED IN THE DETECTION OF ANTIBIOTICS IN MILK WITH HPLC (HIGH PRESSURE LIQUID CHROMATOGRAPHY)

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ABSTRACT

THE CONFIRMATION OF THE COMMERCIAL KITS USED IN THE DETECTION OF ANTIBIOTICS IN MILK WITH HPLC (HIGH PRESSURE LIQUID CHROMATOGRAPHY)

In this study, Charm II Assay was confirmed by HPLC for β-lactam, sulphonamide and tetracycline residues in milk. These antibiotics were chosen because they are most frequently used veterinary drugs and their detection have importance for milk quality and consumer's health.

The results for confirmation of Charm II Assay showed that the test was very sensitive to all groups that were investigated and showed %100 true results for blank samples and spiked samples that were fortified with mixed standards at MRL (maximum residue limit) for each group.

Average recoveries of HPLC used for confirmation were between 47% to 97% for beta-lactams, 61.5% to 84.8% for tetracyclines and 50.4% to 54.6% for sulphonamides.

The results of analysis with the naturally contaminated milk samples showed that Charm II Assay may give false positive results. But this might be because of the high sensitivity of the test that sometimes HPLC may not reach that detection limit of Charm II assay or the milk samples may contain other compounds of investigated antibiotics that HPLC method can not detect.

In samples that were collected for β-lactam determination, only 2 out of 81 samples were detected above MRL where the amounts were 6.5 ppb penicilin-G and 23.8 ppb ampicillin. The MRL for these β-lactam antibiotics are specified as 4 ppb by European Union regulations. The samples investigated for tetracycline residues which were found as positive and confirmed by HPLC were below MRL or negative. In samples investigated for sulphonamides only one sample out of 44 was above MRL where the amount was 119 ppb sulfamethazine.

Analysis with 5 commercial milk samples showed none antibiotic residues. Only 4 samples out of 5 for sulphonamides were screened positive but after confirmation no residues were detected in these samples.

ÖZET

SÜTTEKİ ANTİBİYOTİKLERİ BELİRLEYEN TİCARİ KİTLERİN HPLC (YÜKSEK BASINÇ SIVI KROMATOGRAFİSİ) İLE KONFİRMASYONU

Bu çalışmada, "Charm II Assay" testinin sütteki Beta-laktam, sülfonamid ve tetrasiklin kalıntıları için HPLC doğrulaması yapılmıştır. Bu antibiyotiklerin kullanılmasının nedeni en çok kullanılan veteriner ilaçları olması ve bu yüzden bu antibiyotiklerin saptanmasının süt kalitesi ve tüketici sağlığı açısından önem taşımasıdır.

"Charm II Assay" testinin doğrulama sonuçları testin incelenen antibiyotikler açısından çok hassas olduğunu göstermiştir. Kör numunelerde ve MRL düzeyinde yüklenmiş numunelerin doğrulanmasında 100% doğru sonuç vermiştir.

Doğrulama için HPLC ile elde edilen geri kazanım sonuçları β-laktam için 47% - 97%, tetrasiklin için 61.5%-84.8% ve sulfonamid için 50.4%-54.6% değerleri arasında bulunmuştur.

Doğal olarak kontamine olmuş örneklerde yapılan analizler Charm II testinin yanlış pozitif sonuç verebileceğini göstermiştir. Fakat bu sonuç testin çok hassas olmasından ve bazen HPLC aletinin testin saptama limitine ulaşamamasından kaynaklanabilmektedir ya da sütte HPLC metodunun tespit edemediği gruptaki başka bir antibiyotik çeşidinin bulunduğu tahmini yapılabilir.

Beta-laktam tayini için toplanan 81 örnekten yalnızca ikisi MRL üstünde saptanmıştır ve saptanan miktarlar sırasıyla penicilin-G için 6.5 ppb ve ampicillin için 23.8 ppb'dir. Bu antibiyotikler için MRL 4 ppb dir. Tetrasiklin kalıntıları için araştırılan numuneler arasında pozitif çıkan örnekler HPLC de doğrulaması yapıldıktan sonra sonuçlar MRL seviyeleri altında bulunmuştur ya da hiç bulunmamıştır. Sulfonamid tayini için toplanan 46 örnekten yalnızca birinde MRL üzerinde bir değer bulunmuştur, bu değer is 119 ppb sulfomethazinedir.

5 Market süt örneği ile yapılan analizlerde hiçbir antibiyotik kalıntısına rastlanmamıştır. Sadece sülfonamid grubu Charm II Assay testinde 4 tane pozitif sonuç vermiş fakat örneklerin HPLC de doğrulaması yapıldıktan sonra hiçbir kalıntı tespit edilmemiştir.

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CHAPTER 1

INTRODUCTION

Rapid methods for the detection and characterisation of chemical and veterinary drug residues in foods of animal origin constitutes a dynamic area in food processing, from the stand point of food safety. Residues from these substances are present in edible tissues, milk and eggs and may exert different levels of toxicity on consumers upon consumption (Suhren, et al. 1996). Residual antibiotics in milk can seriously affect consumer's health causing allergic reactions and developing resistant strains. Futhermore, antibiotic contamination in milk can also cause significant economic losses for producers and manufacturers of milk and milk products (Riediker, et al. 2004).

Veterinary and chemical drugs with anabolic effects are used for therapeutic and prophylactic purposes in order to improve breeding efficiency. Although most of them are banned in the European Union they can only be administered in specific circumstances (therapeutic purposes) under strict control. In general, these substances are added to act as growth promoters, improving feed conversion efficiency and increasing the lean to fat ratio (Suhren, et al. 1996). When an antibiotic is used in an animal as a veterinary medicine, the animal can not be marketed or the milk can not be sent for human consumption unless the specified withdrawal period has expired (Riediker, et al. 2004).

Thus, in view of above stated problems easy, rapid and sensitive tests for the determination of these residuals are essential for an effective on line use. Therefore this study is presenting a comparison of the rapid detection method using Charm II kits with the confirmatory analysis of HPLC using milk samples. The results of this study will be informatory with respect to the reliabilty and accuracy of these rapid kits in the detection of the antibiotic residuals in milk samples.

CHAPTER 2

ANTIBIOTICS AS VETERINARY DRUGS

Veterinary medicines are mostly administered to animals in order to treat disease, protect their health and as dietary supplement (Boxall, et al. 2002, Schenck and Callery 1998). Animal drug residues found in milk are a major health and regulatory concern. These drugs are mainly sulfa drugs, sulfamethazine and antibiotics known as penicillin and tetracycline (Hui 1993b). They are administered orally as feed additives or directly by injection. The use of antibiotics may result in drug residues in the milk, especially if not used according to label directions. The antibiotic residues in milk may cause allergic reactions in sensitive individuals, inhibit the growth of starter cultures in the production of cheese and other dairy products, or indicate that the milk may originate from an animal with a serious infection (Schenck and Callery 1998).

Antibiotic residues enter the milk supply chain at farm level. Therefore, it is important that producers realize the factors that lead to antibiotic residues in milk and how these residues can be avoided. Furthermore, the milk testing program should become a component of the quality control process centred on the farm, measuring the success of the industry in producing high quality milk and not being a regulatory program looking for flawed products (Inge and George 2006). The usage of antibiotic varies from country to country, within a country, and between farms, depending on policies. Moreover, the systems used to detect antibiotics in EU countries are developed and implemented by governments, companies and farmers exhibiting many differences. (Inge and George 2006).

2.1. Benefits and Risks of Antibiotics

Antibiotics are added to animal feed at low doses (less than 200 ppm) for two main reasons. Firstly, they are known to increase the growth rate and improve the feed utilization. Secondly, they are known to reduce mortality and morbidity from subsclinical infections by preventing common animal diseases. How exactly antibiotics promote growth and increase feed efficiency is not well known (Jones 1992).

Almost 90% of all antibiotics used in farm animals and poultry are reported to be administired at subtherapeutic concentrations. About 70% of this is for the purpose of disease prevention and 30% are for growth promotion (Sawant, et al. 2005). Antibiotics have major effect in unsanitary environments. Their use controls the spread of infectious disease in crowded conditions. Diseases controlled by the usage of antibiotics include dysentery, mycoplasma and pneumonia. It is predicted that without the usage, the frequency of these diseases would dramatically increase (Jones 1992).

All antibiotics are capable of producing toxic effects, depending on the dose, the time of exposure and the mode of administration. To minimize human exposure to antibiotics from feed, prescribed withdrawal periods are required to be followed by the animal producers. No residues should remain in milk or meat if the required drug withdrawal schedule is followed (Jones 1992). Following the withdrawal times guarantees the safety of milk and milk products for the consumer however may lead to economic losses for the farmer (Heeschen 1991). Withdrawal times may vary for particular drugs, dosage, duration and species.

The extensive use of antibiotics led bacteria to develop defense mechanisms against antibiotics (Sawant, et al. 2005). Residues in milk should be avoided since milk from treated cows may contain large number of potential pathogens and there might be biologically active metabolites or unchanged drugs in the milk causing an adverse effect to the consumer (Concon 1988).

Antibiotic-resistant bacteria are transferred to humans by direct contact with animals fed with antibiotic containing feed or by persons harboring antibiotic-resistant bacteria (Concon 1988).

2.2. Sources of Contamination

The normal and predominant source of milk contamination with antibiotics is the intramammary application of the spesific antibiotic, where untreated quarters may be contaminated via blood circulation or diffusion. FDA surveys have shown that the main reason for residues in milk supply is the illegitimate use of drugs to treat mastitis in animals (Heeschen 1991).

Percutaneous, intrauterine, subcutaneous, intramuscular and intravenous application of antibotics are the other ways of secretory milk contamination.

Contamination can also occur during milking where the inner surface of the parts of a milking machine are rinsed after milking of treated cows milk with untreated cows (Heeschen 1991).

2.3. Classfication of Antibiotics

Antibiotics are categorized according to their chemical structures as shown in Table 2.1 with special reference to theraphy in lactating cows.

Table 2.1. Antibiotics Classified According to Chemical Structure
(Source: Heeschen 1991)

GROUP	INTERNAL GROUP	REPRESENTATIVES WITH PRACTICAL IMPORTANCE
Carbohydrate antibiotics	1.Aminoglycoside antibiotics 2.Other(N- and C-) glycosides	Streptomycin Neomycin
Macrocyclic lactone (lactam) antibiotics	1.Macrolide antibiotics 2.Polyene antibiotics 3.Macrolactam antibiotics	Erythromycin Amphotericin Oligomycin
Quinone and similar antibiotics		Tetracyclines
Amino acid Peptide antibiotics		Penicillins, Cephalosporins, Bacitracin, Polymyxins
Nitrogen-containing Heterocyclic antibiotics	1.Non-condensed (single) heterocycles 2.Condensed (fused) heterocycles	No practical importance
Oxygen-containing Heterocyclic antibiotics	1.Furan derivatives 2.Pyran derivatives	No practical imporance

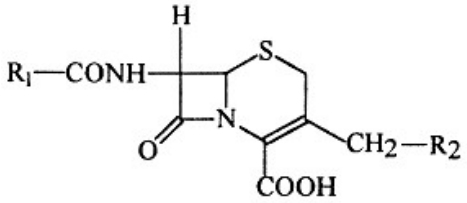
(cont.on next page)

Table 2.1. Antibiotics Classified According to Chemical Structure (cont.)

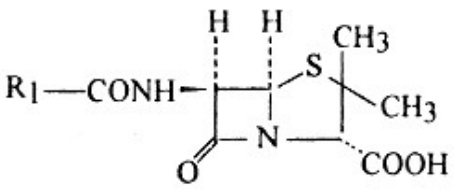
Alicyclic antibiotics	1.Cycloalkane derivatives	
	2.Small terpenes	Streptovitacins
	3.Oligoterpene antibiotics	
Aromatic antibiotics	1.Benzene compounds	Chloramphenicol
	2.Condensed aromatic comp.	Grisefulvin
	3.Non-benzene aromatic comp.	Novobiocin
Aliphatic antibiotics	1.Alkane derivatives	Varitin
	2.Aliphatic carbocyclic acid derivatives	

2.3.1. Beta-Lactam Antibiotics

The Beta-Lactams are the oldest and mostly used antibiotics among all others (Ghinidi, et al. 2002). Beta-lactam group of antibiotics are used especially to fight mastitis which is a serious disease that causes considerable economic losses in world's industry (Riediker, et al. 2004). The widely used antibiotics such as penicillins and cephalosporins are the most important ones (Shammsipur, et al. 2002). As shown in figure 2.1 penicillins and cephalosporins have both beta- lactam ring where in the case of penicillins it is fused to a five-membered thiazolididine ring, and in the case of cephalosporins it is fused to a six-membered Δ^3-dihydrothiazine ring (Fagerquist and Lightfield 2003).



Cephalosporin



Penicillin

Figure 2.1. Structure of Penicillin and Cephalosporin
(Source: Moats and Romanowski 1998)

Beta-lactam ring in antibiotics of this group makes them chemically reactive with the instability of its carbonyl group towards nucleopilic attack. Bacteria produce enzymes that catalyze the hydrolysis of the beta-lactam ring to defend against most penicillins by deactivating them. Cephalosporins are less sensitive to catalytic degradation however penicillinases are classified as penicillinase-resistant or 'penase'resistant (Fagerquist and Lightfield 2003). Classes of beta lactams with bulky side chains are attached to the 6-amino penicillanic acid (6-APA) or 7-amino cephalosporinic acid nuclei, respectively. Because of their antibacterial activity against both gram-positive and gram-negative organisms penicillins are used extensively. However, penicillins produce different degredation products especially in organic solvents because of its limited stability (Shammsipur, et al. 2002). The most frequently tested groups in milk quality assurance programs are penicillins and other beta-lactam antibiotics in the world. Although other antibiotics and chemotherapeutic are available to cure infections in lactating cows, the major problems encountered by the dairy industry are caused by penicillins (Gustavsson, et al. 2002). Essentially, penicillins are not very toxic but in sensitized individuals it can cause strong allergic reactions (Grunwald and Petz 2003). Ampicillin is a widely used semi-synthetic penicillin-like drug. Among the substances of ampicillin are 6-APA, phenyglycine (PhG), penicilloic acid (PA), penilloic acid and ampicillinyl-D-phenyglycine the most significant ones. Determinations of ampicillin have critical importance since the presence of degradation and PhG and 6-APA may decrease activity of ampicillin and casue some side effects and allergic reactions in human body (Shammsipur, et al. 2002). The antimicrobial activity caused by ampicillin can be extended to include gram-negative bacteria such as *Haemophilis influenzae, Escherichia coli* and *Proteu mirabilis.* As high as 10% of over sensitive reactions are observed using this group of antibiotics. Transfer of antibiotics to milk and meat products by and from animals are increased as they are used to fight bacterial infections in various domestic animals (Uslu and Biryol 1999). Maximum residue limits for beta-lactams are presented in Table 2.2.

Table 2.2. MRL's for Beta-lactams
(Source: Popelka, et al. 2004)

Antibiotics	MRL(ppb)
Penicillin G	4
Ampicillin	4
Amoxycillin	4
Cloxacillin	30
Dicloxacillin	30
Oxacillin	30

Lowest tolerance limits in EU among all the antimicrobials belong to beta-lactam antibiotics (Ghinidi, et al. 2002). Maximum residue limits (MRLs) for beta-lactams and other veterinary drugs have been set by the European Union for animal producing food. For example, the MRL is 4 $\mu g\ kg^{-1}$ (4 ppb) for benzylpenicillin and ampicillin in milk. The food industry and the respective authorities carry out control programs and monitoring for drug residues in food for the good of public health and to avoid financial loss (Cacciatore, et al. 2004).

2.3.2. Sulphonamides

The sulphonamide drugs that are used in animal production are soluble in polar solvents such as ethanol, acetone, acetonitrile and chloroform but insoluble in nonpolar solvents. This group has wide variety of polarity with amphoteric properties (pK 4.6-11.5) due to the the basic character of the para-NH_2 group and due to N-H linkage adjacent to the sulphonyl group (Nollet 1992). *p*-aminobenzenesulpone moiety is a part of many sulphonamides which reveals antimicrobial activity. In veterinary practice sulphonamides have been benefited as antibiotic agents in veterinary practice for several decades and are the fifth most widely used group in veterinary antibiotics in European Union countries, accounting to 2% of sales in 1997 (Boxall, et al. 2002, Van Rhijn, et al. 2002). Sulphonamides show antimicrobial activity with tri-methoprim, that is why they are frequently co-administered with this compound. Among many sulphonamides that has been defined, only few are approved for animals as veterinary medicine. The most frequently used sulphonamides are sulfadiazine, sulfadimidine,

sulfamethoxazole, sulfadoxine and sulfadimethoxine. Within the EU, the maximum residue limit in milk has been determined to be 100 ppb. Some countries does not approve sulphonamides in food for human consumption and determination of sulphonamides requires methods that have low detection levels (Van Rhijn, et al. 2002).

Sulphonamides like sulfamethazine (SMZ) and sulfadimethoxine (SDM) which are used improperly in lactating cows is a big concern. The residues of these antibiotics participate in milk which is an important component in the diets of young growing children and adults everyday. Indeed, it was proved that SMZ is a potential carcinogen which raises major concerns (Ko, et. al. 2000, Furusawa 2000).

Sulfamethazine is used therapeutically to treat infections, to control the spread of diseases as preservative, to expand feed fertility and to increase growth rate. The withdrawal time for SMZ is estimated to be fifteen days (Ko, et. al. 2000, Furusawa 2000).

The maximum residue limits of some sulphonamides are given on Table 2.3

Table 2.3. MRL's for Sulphonamides
(Source: Van Rhijn, et al. 2002)

Antibiotics	**MRL(ppb)**
Sulfamethazine	100
Sulfadimethoxine	100
Sulfamerazine	100
Sulfathiazole	100
Sulfamethoxazole	100
Sulfanilamide	100
Sulfadiazine	100

2.3.3. Tetracyclines

Tetracycline antibiotics are close derivatives of the polycyclic naphthacene carboximide. Some of them are product of bacteria called *Streptomyces,* whereas others are semisynthetic products. They are largely used all over the world as oral parenteral medications and as addivites in feed for animals promoting food production due to its activity towards both gram-positive and gram-negative bacteria (Nollet 1992).

Polar functional groups are situated in tetracycline molecules. They have three different dissociation constants, the acidic hydroxy group (pK about 3.3), the dimethylamino group (pK about 7.5), and the hydroxy group (pK about 9.4). Tetracyclines exist as bipolar ions in aqueous solutions at pH 4-7. When the pH increases to 8-9 a marked dissociation of the dimethylamine cation occurs. Their stability is also a critical point. Most of them are photosensitive compounds with reversible epimerization over the pH range of 2-6 (Nollet 1992).

Tetracyclines are used routinely in veterinary medicine for prevention and control of mastitis where they are re-added at subtherapeutic levels to caddle feeds (Schenck and Callery 1998, Cinquina, et al. 2003). Only chlorotetracycline and oxytetracycline are licensed among 10 antibiotic compounds as growth promoters for livestock in the USA (Meyer, et al. 2000). Tertracyclines have an extensive antibacterial spectrum and bacteriostatic activity. They also have a good activity against acute disease caused by gram-positive and gram-negative, which includes the species of *Spirochete, Actinomyces, Ricketsia* and *Mycoplahesma*. The use of these drugs against infectious diseases has become a critical problem, as their residues in milk or meat can be directly toxic or else cause allergic reactions in some hypersensitive individuals. Even more important, consuming of the food that includes low levels of tetracyclines for long periods can cause the spread of drug-resistant micro-organisms (Cinquina, et al. 2003). MRL's for tetracyclines are shown in Table 2.4.

Table 2.4. MRL's for Tetracyclines
(Source: Cinquina, et al. 2003)

Antibiotics	**MRL(ppb)**
Tetracycline	100
Chlorotetracycline	100
Oxytetracycline	100
Doxycycline	100

CHAPTER 3

DETECTION OF ANTIBIOTICS IN MILK

3.1. Introduction

There are various chemical, microbiological and immunological assays used to detect antibiotic residues in milk. Among chemical methods are high-performance liquid chromatography (HPLC), gas-liquid chromatography, radioimmunoassay, thin-layer chromatography (TLC) and electrophoresis (Ramirez, et al. 2003).

Two steps are followed for the analysis to detect antimicrobial residues in milk. Firstly, an enzymatic or microbial or receptor-based method is used as screening tool. Second, the positive samples which contain antibiotic residues are confirmed by a chemical method. As a general rule, confirmatory analysis should identify the compound which is being investigated and to quantitate it. As a confirmatory method for antibiotic residues UV detector is used with high performance liquid chromatography. Because of its low sensitivity and selectivity many purification steps are needed to perform this method. Sometimes, to achieve higher sensitivity, a derivatization step is added to detect the analytes through a flourescence detector. The method takes long time because of purification steps and it is not adaptable for large number of samples (Ghinidi, et al. 2002).

Highly sensitive and selective method can be applied to detect residues in milk with decreasing purification steps if liquid chromatography is coupled with mass spectrometry. Some methods that have been developed for antibiotic residue determination in milk does not exhibit enough sensitivity required by the tolerances set by the European Union Regulation 2377/ 90 (Ghinidi, et al. 2002).

The proper choice of antibiotic screening test plays an important role in the effectiveness and accuracy of residue detection. Screening tests are used to prevent the introduction of the contaminated milk into food chain and, therefore they are frequently used by regulators and food producers (Popelka, et al. 2004). Screening tests can decrease the danger of residue contamination at violative levels if they are reliable to detect them at the concentrations found in bulk and tanker truck milk (Andrew, et al.

2005). Even though their performance are not understood so well, they still are important and necessary part of a farm total quality management program (Sischo 1996).

3.2. High Performance Liquid Chromatography in Residue Analysis

HPLC usage is increasing day by day in the field of residue analysis. The variety of mobile phases, the extensive library of column packings and the variation in modes of operations are the reasons for this method to be in demand. HPLC have progressed for determination analysis in food industry after all these advantages combined with various types of detectors available. In residue analysis of edible animal products, the sample often has much higher concentrations of endogenous interfering components but a very low content of residues. It is necessary to access variety of producers for isolations, derivatization and quantitation of the compound of interest since the nature and concentration of these components can vary widely (Nollet 1992).

Sample deproteinization is the first step in animal originated food residue analysis. Mineral or organic acids like hydrochloric or trichloroacetic acid and/or water-miscible organic solvents such as acetonitril, acetone or methanol, which precipitate the proteins and allow their removal by centrifugation are used frequently. Sample deproteinization helps releasing protein-bound residues besides protecting the HPLC column from irreversible contamination. In most conditions analyte extraction into a solvent is the second step where extraction efficiency is determined by the polarity of the extracting solvent, the pH of the sample/solvent system and the sample-to-solvent volume ratio. Extract clean-up process is usually involved as the third step in sample preperation. The easiest procedure is a simple liquid-liquid partitioning between two immiscible solvents, where the analyte is selectively partitioned in one of the two phases (Nollet 1992).

HPLC analysis of antimicrobial residues is mainly performed in either reverse-phase mode or in the ion exchange mode. The efficiencies in the ion exchange mode are determined to be lower than those obtained by normal-or-reverse-phase HPLC. Usually excessive tailing due to the inhomogeneity of the absorbent surface is obtained. Many parameters can influence both the resolution of the compounds and column efficiency in reverse-phase HPLC. In order to obtain best results a combination of the appropriate

stationary/mobile phase system and the mode of elution (isocratic or gradient) must be determined. Alkyl-bonded (C_8, C_{18}) stationary phases are used with mobile phases such as methanol or acetonitrile. The content of the organic modifier in the mobile phase is a function of both the polarity of the analyte and the type of column packing (Nollet 1992).

For residue analysis fluorescence detection has been proved to be valuable tool where interferences from food components must be reduced or eliminated. Fluorescent derivatives of many non-fluorescing solutes emerging from the chromatographic column can be prepared using specific fluorescence-labeling reactions. Comparing retention times is the key for identification of eluted compounds with reference compounds processed in an identical manner. Sometimes retention times are not enough by itself since a retention time can be observed for more than one compound or several components can be eluted at same retention time and chromatograph may show only one peak. Repeating the sample analysis on a different packing material can contribute to more satisfying results (Nollet 1992).

3.3. Rapid Test Methods for Antibiotic Residues

Milk that contain antibiotic residues must be discarded. In the last years, the number of tests available has increased for detecting penicillin and other common antibiotics . There are some tests that are both qualitative and quantitative and some of them can be applied to detect antibiotics before they enter the milk supply at the source (Hui 1993a).

More purified and improved drugs are being used to treat cattles, because of this detection methods for residues are being refined and improved (Hui 1993a). Rapid tests were designed in view of the needs of milk processors. These tests are simple and suitably sensitive and take very short time (10-20 minutes) to complete. The cause of the desire to shorten the duration times expedite the development of enzymatic and immuno/receptor tests. In the 1980's rapid detection tests were presented for the first time. These methods are more expensive than microbiological methods but their major insufficiency is that only materials that react with immobilized receptor can be detected, e.g., the beta-lactams (Zvirdauskiene and Salomskien 2007).

Milk producers have to be sure that the milk they supply is free from the list of antibiotics that are prohibited, or that the levels of antibiotics are lower than maximum residue limits (MRL). There is no microbial inhibitor test that can detect all substances at the MRLs set by European Union Regulations. Most of the methods are targeted to beta-lactam for the reason that they are most commonly used veterinary drugs in the theraphy of cows in many countries. Enzymatic tests such as Penzym test, Penzym S (UCB Bioproducts, Belgium) and immunological tests such as Delvo-X-Press β-Lactam (DSM, Netherlands), β-STAR (UCB Bioproducts, Belgium), ROSA test (Charm Sciences, Inc., USA) are the most widely used rapid tests for antibiotic residues detection in milk (Zvirdauskiene and Salomskien 2007).

3.3.1. Bacterial Growth Inhibition Methods

The inhibition of growth of responsive microorganisms were the mechanism of first methods to detect antibiotic residues in milk. A cyclinder plate assay method and filter paper disc method were used in the early 1940's. At first, *Bacillus subtilis* was used as responsive microorganisms but in recent years, methods have started to rely on *Bacillus stearothermophilus* inhibition. These assays are specific for Beta-lactams but most have been developed for penicillin detection. Delvotest SP (DSM, Netherlands), Copan Test (Copan, Italy), Charm Farm-960 Test (Charm Sciences, Inc., USA) are the most commonly used microbial inhibitor tests which use spores of *Bacillus stearothermophilus* var. *Calidolactis* (Zvirdauskiene and Salomskien 2007). The principle for this tests is comparing clear zones on an agar plate medium to which bacterial spores have been seeded. Zones that belong to sample is compared with the zones of known amount of penicillin for quantitative determinations. Sensitivity and reproducibility of the method is affected by the depth of agar, where a thin layer is more sensitive than a thick layer (Hui 1993a).

Acid production during growth of *B. Stearothermophilus* var. c*alidolactis* is utilized to develop commercial Delvotest SP (DSM, Netherlands). If inhibitors are absent, the bacteria grow and produce acid, a change is seen in the indicator. Test kits are available for individual as well as for multiple sample analyses. A commercially available test kit BR TEST AS detects a host of inhibitory substances. Agar diffusion and color reduction techniques are combined in this method using *B. Steathermophilus*

var. *calidolactis* spores. During incubation, the metabolism of the bacteria is inhibited if drug residues are in the excess of the detection limit of the method. Test color remains blue if inhibitors are present whereas during incubation of inhibitor-free milk, oxidation-reduction reactions change the color to yellow. This test is appropriate for raw and pasteurized milks (Hui 1993a).

3.3.2. Competitive Binding Methods

Various test procedures are developed by Charm Sciences, Inc.(Malden, MA) to detect inhibitory substances in milk. The original test, Charm Test, has been recasted a few times over the years to make its sensitivity and accuracy better and expand its selectivity. Final action procedure was developed for assay of beta-lactams in milk in 1984. In this procedure the principle is that beta-lactam residues have a specific, irreversible propensity for enzyme sites on the cell wall of microorganisms. 14C-labeled penicillin and *Bacillus stearothermophilus* vegetative cells are used for this method. If penicillin is present in the sample, it competes to bind enzyme sites on the bacterial cell wall and more 14C-label remains free in the solution. Positive and negative controls are prepared before sample analysis and results of these controls are compared to the sample within 15 minutes (Hui 1993a). Charm II procedure are being used by many dairy laboratories where seven families of antimicrobial drugs can be screened. Necessary binding sites are procured by two different microorganisms for the seven drug families. Beta-lactam, tetracyclines, macrolides, streptomycin, novoiocin, sulphonamides and chloramphenicol can be counted as these families. Biologically active drugs are detected in about 8 minutes for one or two families or 15 minutes for all seven families. Reagents are in tablets and single tests can be performed easily. Sensitivity of this method is good for β-lactam antibiotics and all sulfa drugs in raw milk, milk powder and pasteurized milk (Hui 1993a). Figure 3.1 gives a basic idea for the principle of Charm II Assay test. The sample is incubated with a binding agent and a tracer which contains labelled version of the antibiotic to be detected. The antibiotic residue in milk compete with this labelled antibiotic for the receptors on binding agent. A scintillation counter measures the amount of tracer on the binding agent and compares with a control point (Hall, et al 2003)

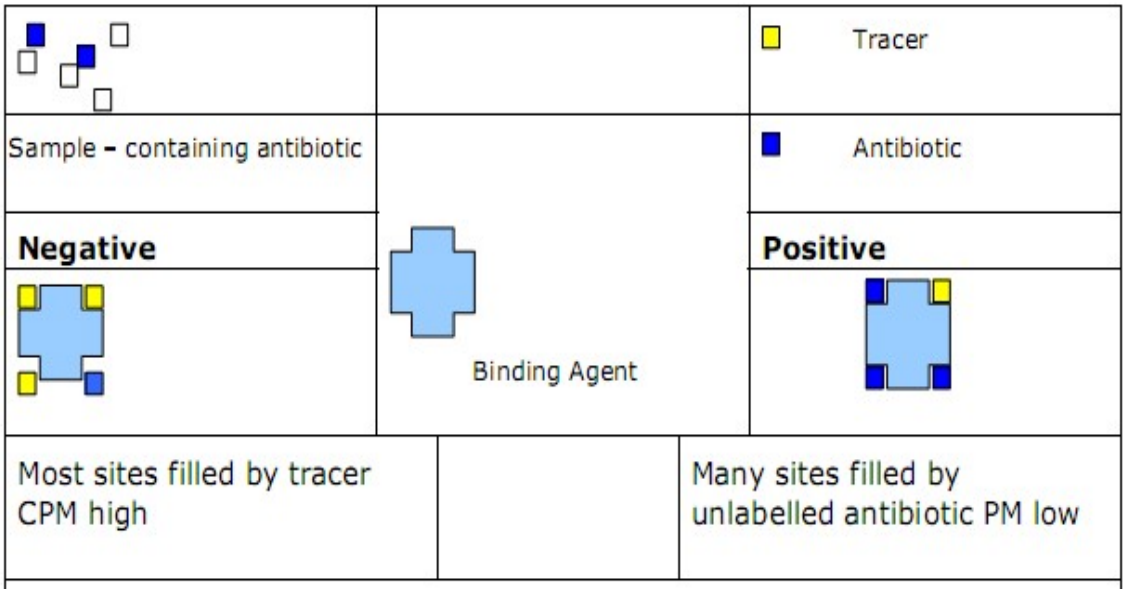


Figure 3.1. Charm II Assay Test Procedure
(Source: Hall, et al. 2003)

The binding of DD-carboxypeptidase to beta-lactam antibiotics is utilized for another competitive binding method (Hui 1993a). The Penzym-test is a rapid enzymatic test to detect beta-lactam antibiotics. The principle of the test is that β-lactam antibiotics inhibit the activity of DD-carboxypeptidase which liberates D-alanine from an enzyme substrate. Color change is the proof for the antibiotic presence. If antibiotics exist in the sample, D-alanine can not be liberated and no color change is observed. The test produces a yellow color if the sample is positive. This test is available in a kit and each of them should be checked before the use with penicillin standards, as the test detects beta-lactam residues at 0.01 IU/ml in raw milk. Positive and negative controls should be prepared for all samples. Results are ready in 20 min ((Neaves 1999, Suhren, et al. 1996)

3.3.3. Other Methods

An immunological agglutination technique called the Spot test is used to detect antibiotics in milk. Milk samples are mixed with the Latex beads coated with specific inhibitory molecules (penicillin-G, cephapirin, cloxacillin) and antibodies attached inhibitory molecules. In the case of inhibition in the milk, the antibody or inhibitor-coated latex beads do not agglurinate (Hui 1993a).

Enyzme-linked immunosorbent assays (ELISA) are expeditiously becoming preferable to detect specific antibiotics in food. In this assay immobilised antibodies capture the target antibiotic groups where a competition between the target antibiotic group and the internal antibiotic standard is observed. The antibody-antibiotic complex is linked to an enzyme that cause a color reaction. The presence or absence of antibiotic or drug residues are proved by color changes. Lactek screening kit, CITE probe kit, SIGNAL detection test, EZ-SCREEN and Agri-Screen methods are being applied to milk at present. They all have some advantages and disadvantages and must be considered according to specific requirements for the analysis (Hui 1993a, Neaves 1999).

CHAPTER 4

MATERIALS AND METHODS

All milk samples that were used in the experiments were collected by Bornova Veterinary Control and Research Institute. These samples were supplied between September 2006 and June 2007. Collected samples were centrifuged and stored in freezer at 0 º C until used for analysis. All the Charm II Assay kits together with scintilliation fluid for each antibiotics (β-lactams, tetracyclines and sulphonamides) were partly donated and purchased by Maysa Inc. Turkey.

4.1. Determation of Beta –Lactam Residues

4.1.1. Chemicals and Reagents

Standards of beta-lactam antibiotics (ampicillin, penicilin G, oxacillin, cloxacillin, dicloxacillin) were obtained from Reidel-de Haen and Applichem with a purity higher than 97%. Each stock of standard solutions (1000 ppm) was prepared in pure water and stored in a refregirator. Methanol, isooctane and acetonitrile were of analytical-reagent grade (Merck). Ultra-pure water was obtained with a Milli-Q ultrafiltration unit from Millipore (Molsheim, France). Monobasic sodium phosphate dihydrate, dibasic sodium phosphate dihydrate, sodium thiosulfate pentatrydrate, hydrogen sulfate tetraburyl ammonium, benzoic anhydride, sodium hydroxide and sulfuric acid were obtained from Merck.

4.1.2. Equipments

Centrifugation was performed with a refrigerated centrifuge model G R 4.11 (Jouan). Strata-X polymeric sorbents were used for solid phase extraction. High pressure liquid chromatography (HPLC) consisted of HPLC Pump model SP8800 (Spectra Physics) equipped with an autosampler (Spectra Physics type 8775) fitted with 200 µl loop and 2.5 ml syringe, an analytical column C8 (250 x 3 mm; 5µm) (type

symmetry Waters), a guard column RP18e (4x4 mm; 5 µm) (Merck) and a UV absorbance detector model Spectroflow 773 (Kratos). Intergrator model was SP4290 (Spectra Physics). Charm II 6600 Analyzer were used for Charm II analysis throughout the whole study for the determination of each antibiotic (ampicillin, penicilin-G, oxacillin, cloxacillin, dicloxacillin).

4.1.3. Charm II Assay Procedure

Charm II Assay kits include two tablets which have different colors (green and yellow). These tablets consist of binding agent and tracer antibiotic. Tracer tablet (green) of Charm II Assay was added into centrifuge test tube to which 300 µl pure water was added and mixed for 10 seconds. Afterwards 5 ml milk sample was added and mixed again. This suspension was incubated at 65 ºC for 2 minutes. Afterwards, binding reagent tablet (yellow) was added, mixed and followed with incubation at 65 ºC for 2 minutes. Incubated samples were centrifuged for 3 minutes at 3300 rpm to which afterwards 300 µl pure water was added and mixed for another 10 seconds. Finally, 3 ml of scintillation fluid was added and counted on scintillation counter.

4.1.4. HPLC Procedure

4.1.4.1. Preparation of the Calibration Curve

Intermediate standard solutions (10 ppm) of each compound were prepared from 1000 ppm stock standard solutions. From these intermediate standard solutions, 1 ppm standard working solution was prepared with phospate buffer at pH 8. Calibration solutions containing 20, 40, 80 and 160 ppb of each compound was prepared in phosphate buffer. 50 µl of 0.2 M benzoic anhydride was added to the solutions and kept in hot water bath at 50 ºC for 3 minutes. After that, 500 µl derivatizing agent was added. These were kept in hot water bath at 65º C for another 10 minutes. Afterwards, solutions were cooled to room temperature in cold water bath in the dark. 100 µl of solutions were taken for injection to HPLC.

4.1.4.2. Sample Preparation

Fortified or incurred milk sample (5 ml) was mixed with 30 ml of extraction solution in a centrifuge tube adjusted to pH 4 with 2 N sulfuric acid. This suspension was centrifuged for 10 min at 4000 rpm at 4 ºC. The aqueous phase was transfered into a clean centrifuge tube of 50 ml and adjusted to pH 8 with 5 M sodium hydroxide. Afterwards this suspension was centrifuged for 5 min at 4000 rpm at 4 ºC. Besides a Strata-X polymer sorbent was placed on the vacuum manifold and the cartridge was washed with 3 ml methanol. This was rinsed with 3 ml ultra pure water. The sample solution was poured immediately into the cartridge and air was drawn into the cartridge for 1 minute. The washes were discarded followed by the cassation of the vacuum and 1 ml elution solution (%50-50 acetonitrile-phosphate buffer at pH 8) was added. The elution solution was soaked in the cartridge for 1 minute and vacuum was set. Finally, samples were eluted at a flow rate of 3 ml/min.

4.1.4.3. Precolumn Derivatization

0.2 M benzoic anhyride solution (50 µl) kept in a water bath at 50 ºC for 5 minutes was mixed with 500 µl derivatizing reagent and incubated at 65 ºC for 10 minutes. This solution was cooled to room temperature in a dark place. Sample vial was prepared and placed on the autosampler equipped with a 200 µl loop and 2.5 ml syringe. The derivatized antibiotic peak area was measured at 325 nm.

4.2. Determination of Sulphonamide Residues

4.2.1. Chemical and Reagents

Acetic acid, acetonitrile, acetone, methanol, n-hexane, chloroform, sulfiric acid and standard of sulfathiazole were purchased from Merck. Standards of sulfamethazine, sulfadimethoxine, sulfamerazine, sulfanilamide, sulfamethoxazole, sulfadiazine were purchased from Sigma. Fluorescamine was obtained from Applichem. Ultra-pure water was obtained with a Milli-Q ultrafiltration unit from Millipore (Molsheim, France).

4.2.2. Equipments

HPLC apparatus used in the analysis was equipped with reversed–phase C_{18} column, 5 µm, 150x4 mm with LC-10 AT VP liquid chromatography, SCL-10 A VP system controller, A DGU-14 A degasser, CTO-10 AS VP column oven, RF-10 A XL flurescence dedector and PCX 5200 post column derivatizer. Charm II 6600 Analyzer were used for Charm II analysis.

4.2.3. Charm II Assay Procedure

Charm II Assay kits include two tablets which have different colors (white and pink). These tablets consist of binding agent and tracer antibiotic. Tracer tablet (white) of Charm II Assay mixed with 300 µl pure water for 10 seconds initally was added to the milk sample (5 ml) and mixed. Following this, binding reagent tablet (pink) was added and sample was mixed and incubated at 85 ºC for 3 minutes. These were centrifuged for 3 minutes at 3300 rpm and 300 µl pure water was added and mixed for another 10 seconds. Finally, 3 ml scintillation fluid was added and counted on scintillation counter.

4.2.4. HPLC Procedure

4.2.4.1. Preparation of the Calibration Curve

Stock standard solutions of sulfamethazine, sulfadimethoxine, sulfamerazine, sulfanilamide, sulfathiazole, sulfadiazine, sulfamethoxazole were prepared by dissolving 10 mg of each compound in 10 ml of methanol to obtain a final concentration of 1000 ppm. Stock standard solutions were stored at -8 ºC - +4 ºC in refrigerator. These solutions were diluted to 100, 200, 300 and 400 ppb to be used in the preparation of the calibration curve.

4.2.4.2. Sample Preparation

Milk samples (5 ml) centrifuged at 4000 rpm for 10 minutes were placed into a seperatory funnel and mixed with 50 ml of extraction solution (chloroform-acetone, 2+1 v/v). Vigorous shaking for 1 minute, each time by vending the stopper, was repeated twice until phase separation was observed. Extraction solution was withdrawn and 25 ml of fresh extraction solution was added and the same procedure as decribed was repated. Similary, the extraction solution was removed after the phase separation. The residual separation solution was evaporated using a rotary evaporator at 32 ºC under vacuum. The residue (2 ml) was dissolved in mobile phase (1% acetic acid) and vortexed, followed by the adition of n-hexane (5 ml). Phases were separated within 2 minutes followed by vortexing another minute. Aqueous layer (bottom) phase was removed and filtered into the autosampler vial after second phase separation .

4.3. Determination of Tetracycline Residues

4.3.1. Chemicals and Reagents

Methanol, acetonitrile, disodium hydrogen phospate dihydrate, oxalic acid dihydrate, citric acid monohydrate were purchased from Merck. Ethylene diamine tetra acetic acid disodium salt, trichloroacetic acid were purchased from Prolab. Ultra-pure water was obtained with a Milli-Q ultrafiltration unit from Millipore (Molsheim, France). Standards of oxytetracycline, tetracycline, chlortetracycline and doxycycline were supplied from Reidel-de Haen.

4.3.2. Equipments

Analyses were carried out on a HPLC–DAD model HP 1100 system. Seperations were carried out by C_{18} Hypersil BDS column (250x 4 mm, 5µm) coupled with a guard column. Centrifugation was performed with a refrigerated centrifuge model GR 4.11 (Jouan). Strata-X polymeric sorbents were used for solid phase extraction.

4.3.3. Charm II Assay Procedure

Charm II Assay kits include two tablets which have different colors (white and orange). These tablets consist of binding agent and tracer antibiotic. Tracer tablet (white) of Charm II Assay mixed with 300 µl pure water for 10 seconds initally was added to the milk sample (5 ml) and mixed. Following this step, binding reagent tablet (orange) was added and sample was mixed and incubated at 35 ºC for 3 minutes. These were centrifuged for 5 minutes at 3300 rpm and 300 µl pure water was added and mixed for another 10 seconds. Finally, 3 ml scintillation fluid was added and counted on scintillation counter.

4.3.4. HPLC Procedure

4.3.4.1. Preparation of the Calibration Curve

Stock standard solutions of oxytetracycline, tetracycline, cloxacycline and doxytetracycline were prepared by dissolving 10 mg of each compound in 10 ml of methanol to obtain a final concentration of 1000 ppm. Stock standard solutions were stored at -20 ºC. These solutions were diluted to give a series of solutions (50, 100, 150, 200 and 250 ppb) to be used in the preparation of the calibration curve.

4.3.4.2. Sample Preparation

Homogenised milk sample (5 ml) was mixed with 2 ml of 20% tricholoroacetic acid (TCA) and shaken. To this solution 20 ml of McIlvaine buffer (13.72 g disodium hydrogenphosphate dihydrate, 11.8 g of citric acid monohydrate, 33.62 g of ethylene diamine tetra acetic acid disodium salt in 1 litre of water) was added followed by centrifugation at 4000 rpm for 20 minutes. Polymeric sorbent cartridge activated with 3 ml methanol and 3 ml of pure water was used for the purification of tetracylines. After passing the samples through these cartridges and washing it with 3 ml of methanol, tetracylines were eluted by using 3 ml acetonitrile. After removing the solvent under the nitrogen stream it was dissolved in 1 ml of 0.01 M oxalic acid and filtered through a 0.45 µm syringe filter. Finally, 200 µl of the solution was injected into the HPLC-

DAD system. 0,01 M oxalic acid dihydrate was used as mobile phase. Samples were analized at 360 nm.

CHAPTER 5

RESULTS AND DISCUSSIONS

5.1. Introduction

In this study two different methods such as Charm II Assay and High Performance Liquid Chromatography (HPLC) were used to determine tetracycline, beta-lactam and sulphonamide residues in milk. The former was used mainly for screening purpose whereas the latter was used for confirmation purpose. In the first part, a validation study was performed to determine whether Charm II Assay was adequate to detect the antibiotics at MRL limits in milk samples. A total of 20 negative and 20 positive samples including the duplicates were fortified at maximum residue limits with mix standards of every group of antibiotics that was investigated and confirmed by HPLC. Besides this study includes the analysis of milk samples that were taken by Bornova Veterinary Control and Research Institue between September 2006 and July 2007 in order to give an overview on antibiotic residues in the milk samples collected from various part of Turkey. Also, five commercial milk samples were purchased from local market and investigated for antibiotic residues.

5.2. Validation of Charm II Assay

For validation of Charm II Assay 5 compounds of beta-lactam antibiotics (penicillin-G, oxacilline, cloxacillin, ampicillin, dicloxacillin), 4 compounds of tetracycline antibiotics (oxytetracycline, tetracycline, chlortetracycline and doxycycline) and 6 compounds of sulphonamide antibiotics (sulfamethazine, sulfadimethoxine, sulfamerazine, sulfanilamide, sulfadiazine, sulfathiazole) were analysed. A total of 20 (including the dublicates) different blank samples and 20 fortified samples for each compound of β-lactams were assayed.

Table 5.1 shows the accuracy rates for blank samples and fortified samples with penicilin-G, oxacillin, cloxacillin, ampicillin and dicloxacillin. Usually accuracy rate lower than % 95 are not considered suitable.

Table 5.1. Accuracy Rates for Beta-lactam Validation

Samples	Accuracy rate
Blank Samples	100%
Oxacillin	100%
Cloxacillin	100%
Ampicillin	100%
Dicloxacillin	95%
Penicillin-G	100%

As it is can be seen in Table 5.1 for only dicloxacillin, one false negative result was observed. Details of counted samples are given in Appendix A.

Table 5.2 shows accuracy rates for samples that were free of sulphonamides and that were fortified at MRL levels with sulphonamide group of antibiotics. No false result was observed for sulphonamides (see for detail counts Appendix B) .

Table 5.2. Accuracy Rates for Sulphonamide Validation

Samples	Accuracy rate
Blank Samples	100%
Sulfametazine	100%
Sulfadiazine	100%
Sulfamerazine	100%
Sulfamethoxazole	100%
Sulfathiozole	100%
Sulfadimethoxine	100%

Usually, one false result is acceptable in 20 samples (inaccuracy rate can not be more than % 5) for every group that are tested with Charm II Assay.

Table 5.3 shows the accuracy rates of tetracyclines using 100 ppb fortified milk samples counted on Charm II Assay. In appendix C results can be seen as detailed.

Table 5.3. Accuracy Rate for Tetracycline Validation

Samples	Accuracy rate
Blank Samples	100%
Doxycycline	95%
Tetracycline	100%
Oxytetracycline	100%
Chlorotetracycline	95%

Validation of doxycycline and chlorotetracycline gave one false negative result. Validation results for all groups were satisfactory.

Table 5.4 summarises the results of blank samples used for detection of antibiotic residues by applying Charm II Assay. If sample count per minute (cpm)/cpm zero is greater than 1.0, the sample is considered as negative. CPM number is a resulting count after analysis of the pellet in a scintillation counter for 1 minute. CPM zero is a control point that was found by zero control standard counting. No false positive result was observed for the antibiotics that were investigated.

Table 5.4. Results of Charm II Assay for Blank Samples Detection

Blank samples	CPM sample/CPM zero	n
Beta-lactam Samples	1.29	20
Sulphonamide Samples	2.11	20
Tetracycline Samples	1.24	20

Similarly, Table 5.5, 5.6, 5.7 show the average cpm sample/cpm zero results and their standard deviations for each group of antibiotic containing samples. It can be concluded that Charm II Assay is able to detect antibiotic residues at maximum residue limits easily.

Table 5.5. Summary of Charm II Assay Results for Beta-lactams

Antibiotic	cpm sample/cpm zero	SD	n
Penicillin-G	0.50	0.095	20
Oxacilline	0.74	0.064	20
Cloxacillin	0.67	0.072	20
Ampicillin	0.79	0.118	20
Dicloxacillin	0.63	0.163	20

Table 5.6. Summary of Charm II Assay Results for Sulphonamides

Antibiotic	cpm sample/cpm zero	SD	n
Sulfamethazine	0.52	0.039	20
Sulfadiazine	0.59	0.032	20
Sulfamerazine	0.57	0.039	20
Sulfamethoxazole	0.49	0.046	20
Sulfathiozole	0.59	0.040	20
Sulfadimethoxine	0.61	0.089	20

Table 5.7. Summary of Charm II Assay Results for Tetracyclines

Antibiotic	cpm sample/cpm zero	SD	n
Tetracycline	0.76	0.100	20
Chlorotetracycline	0.76	0.115	20
Doxycycline	0.74	0.097	20
Oxytetracycline	0.78	0.118	20

5.3. Confirmation of Charm II Assay

5.3.1. Confirmation of Beta-lactam Antibiotics in Milk

For confirmation of β-lactam residues, a total of 20 blank samples and 20 spiked samples including the dublicates tested with Charm II Assay were assayed by HPLC at MRL levels. Table 5.8 shows the results of Charm II Assay for blank samples and Table 5.9 shows the results for fortified samples (here 4 ppb for ampicillin, penicillin-G and 30 ppb for cloxacillin, dicloxacillin, oxacillin were mixed) on Charm II analyzer. All blank samples gave correct results with Charm II analysis as it can be seen on Table 5.8.

Table 5.8. Results of Blank Samples for Beta-lactams on Charm II Assay

Sample Number	Control Point	AverageCPM(n=2)	Results
1	586	924.5	not found
2	586	829.5	not found
3	586	994	not found
4	586	716.5	not found
5	586	858	not found
6	586	874	not found
7	586	820	not found
8	586	713	not found
9	586	661	not found
10	586	696.5	not found

As it can be seen on the Table 5.9 no false positive results were observed for fortified samples. These samples were then confirmed by HPLC. Goodness of fit are shown in Table 5.10 for calibration sets. Calibration curves for beta-lactam can be seen in Appendix D.

Table 5.9. Results of Spiked Samples for Beta-lactams on Charm II Assay

SampleNumber	Control Point	AverageCPM(n=2)	Results
1	834	364	positive
2	834	389.5	positive
3	834	659.5	positive
4	834	465	positive
5	834	453.5	positive
6	834	525.5	positive
7	834	437	positive
8	834	451	positive
9	834	302.5	positive
10	834	455.5	positive

Table 5.10. Goodness of Fit for Beta-lactam Calibration

Beta-lactam	R^2
Penicillin-G	0.9930
Oxacillin	0.9920
Dicloxacillin	0.9969
Cloxacillin	0.9784
Ampicillin	0.9978

Correlation coefficent was between 0.9784 and 0.9978 as it can be seen in Table 5.10. The closer correlation coefficent to 1, the better the calibration curves is. The amount of detected compound is found closer to the real amount if correlation efficient is close to 1.

Figure 5.1 and Table 5.11 show the values and the chromatogram of blank samples for beta-lactam detection. No antibiotic peak was detected by HPLC analysis for all blank samples.

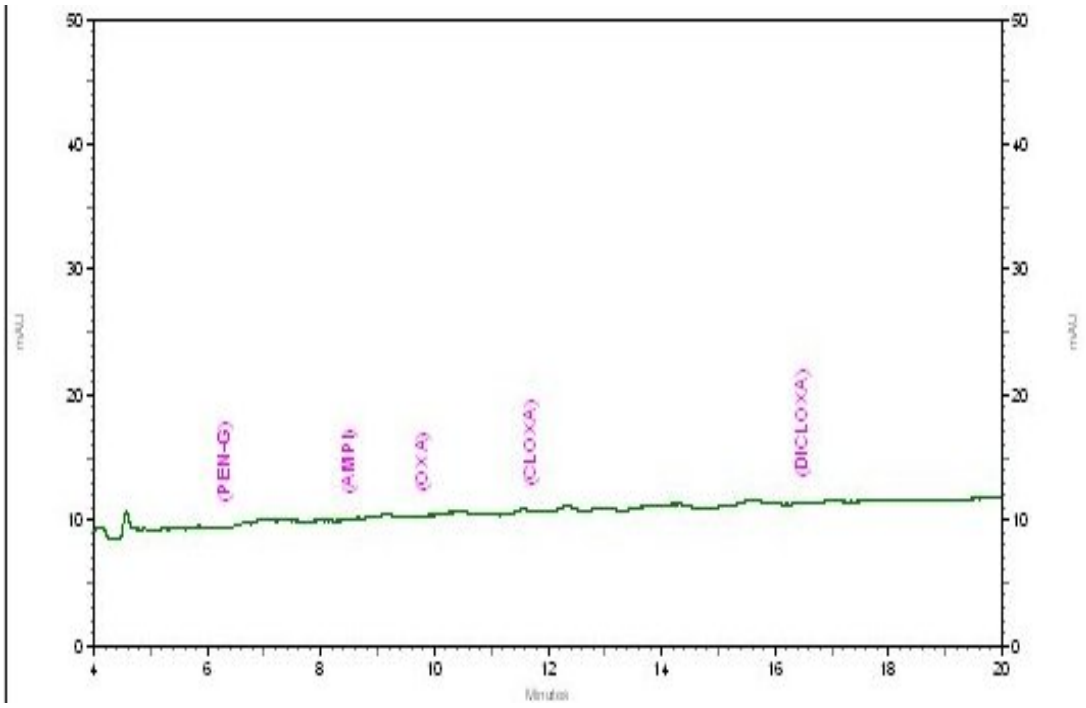


Figure 5.1. Chromatogram Obtained for Beta-lactam by HPLC from Blank Sample

Table 5.11. Concentration of Beta-lactam Antibiotics in Blank Sample

Retention Time	Name	Area	ESTD concentration
	PEN-G		0.00 BDL
	AMPI		0.00 BDL
	OXA		0.00 BDL
	CLOXA		0.00 BDL
	DICLOXA		0.00 BDL
Totals			

A typical chromatogram of penicilin-G, ampicillin, oxacillin, cloaxacillin and dicloxacillin standards at 365 nm is shown in Figure 5.2. Retention times were 6.18, 8.50, 9.80, 11.75 and 16.58 min., respectively as shown in Table 5.12. The standards of antibiotics were separated in 18 minutes with symmetrical peaks.

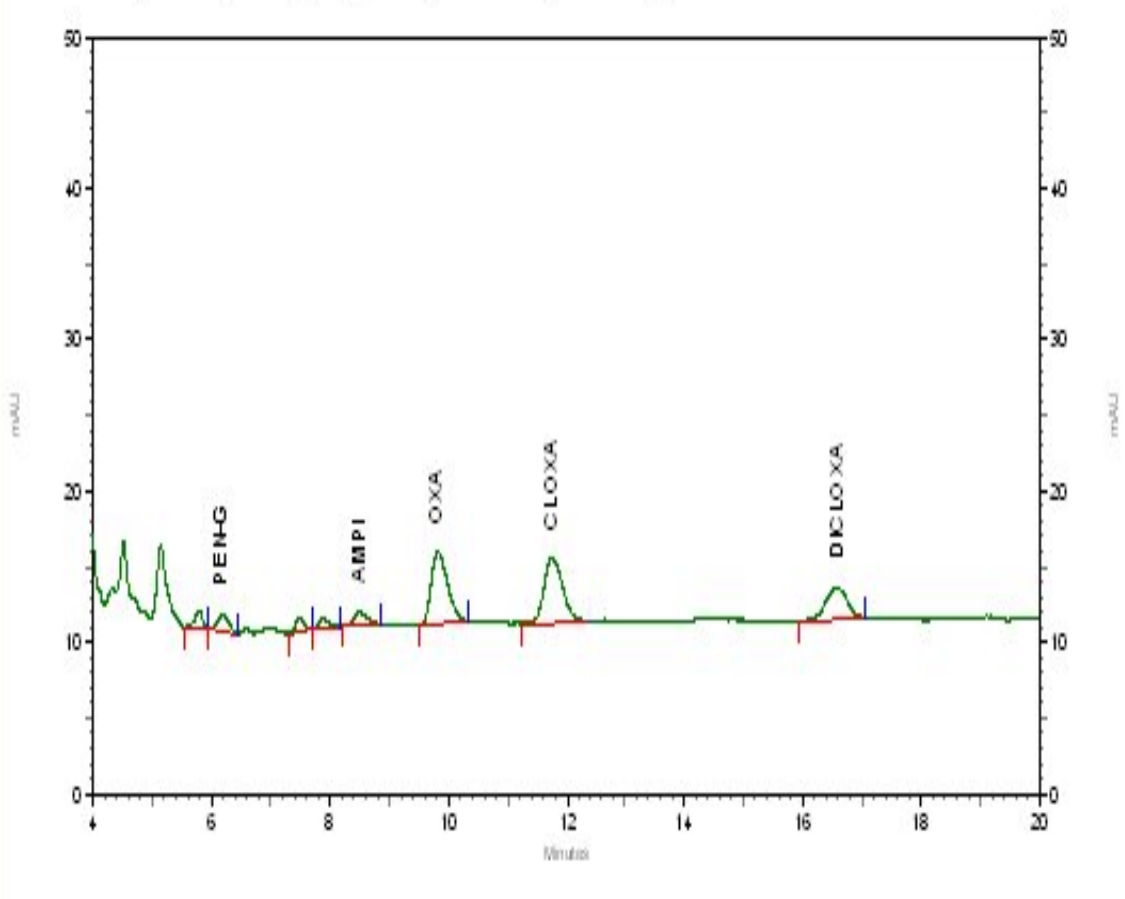


Figure 5.2. Chromatogram Obtained for Beta-lactam by HPLC from Spiked Sample

Table 5.12. Concentration of Beta-lactam Antibiotics in Spiked Sample

Retention Time	Name	Area	ESTD concentration
6.18	PEN-G	14592	12.79
8.50	AMPI	14515	15.33
9.80	OXA	92870	115.05
11.75	CLOXA	95544	114.14
16.58	DICLOXA	54290	84.84
Totals		271811	342.15

In order to verify the specificity of the method, 10 blank samples and 10 spiked samples from different origins tested initially with Charm II Assay were confirmed by HPLC. Penicilin recoveries were the lowest whereas for ampicillin these were the highest. It was confirmed that Charm II Assay didnt give any false result for beta-lactam determination. Average, standard deviation and relative standard deviation of recoveries for 20 spiked samples are given at Table 5.13.

Table 5.13. Average, Standart Deviation and Relative Standard Deviation of Recovery Values of Spiked Samples for Beta-lactams

	Penicilline-G	Ampicillin	Oxacillin	Cloxacillin	Dicloxacillin
Average Recovery (%)	47	97	67	64	62
Standard Deviation (%)	8.2	17.4	13.2	12.2	5.6
RSD(%)	18.9	17.1	21.5	20.5	10.7

The mean of recovery for penicilin-G was 47 (%). The recoveries could be improved for penicilin-G, but the aim of this study was to develop a method in order to detect all five beta-lactam group of antibiotics at the same time.

Penicilins have the strongest absorbance at 210 nm. They normally don not have a very strong UV chromophores because of that they need compherensive purification before liquid chromatography analysis to eliminate milk compounds which also shows strong absorbance (Popelka, et al. 2004). In our study, 325 nm was used for LC analysis therefore penicilin might have shown low absorbance at this wavelength and recoveries for this antibiotic might have been affected by this.

Popelka et al. (2004) reported a similar method for multiresidue determination of beta-lactams. The method consists a derivatization step with benzoic anhydride and 1,2,4-triazole mercuric chloride. Extraction with phospate buffer pH at 9 was followed with purification on Lichrospher C18 column at 325 nm. Amoxicillin, cloxacillin, ampicillin, oxacillin, penicilin-G was detected with this method and penicilin-G recovery was determined as 74%.

Brito and Junqueira (2006) presented a method for determination of ampicillin, penicillin G and penicillin V. Acetic anhydride and 1-methyl-imidazole solution containing $HgCl_2$ were used for derivatization. C18 SPE cartridge was used for sample extraction. The analysis was performed with a C18 column using mobile phase consisting of acetonitrile and phosphate buffer (pH 6.5) at 325 nm. Average recoveries for ampicillin and penicilin-G were between 60.0%-104.9% and 82.7%-109.2% respectively. Our results were below these which indicated that a serious material and method improvement should be considered in future work.

5.3.2. Confirmation of Sulphonamide Antibiotics in Milk

For confirmation of sulphonamide residues, a total of 20 blank samples and 20 spiked samples including the duplicates tested with Charm II Assay were analysed by HPLC. Table 5.14 shows the results of Charm II Assay for blank samples and Table 5.15 shows the results for fortified samples (100 ppb mix for each compound of sulphonamides).

Table 5.14. Results of Blank Samples for Sulphonamides on Charm II Assay

Sample Number	Control Point	Average CPM (n=2)	Results
1	790	1185	Not found
2	790	1171	Not found
3	790	1190	Not found
4	790	996.5	Not found
5	790	907.5	Not found
6	790	888	Not found
7	790	2082	Not found
8	790	1163.5	Not found
9	790	1044	Not found
10	790	860	Not found

Table 5.15. Results of Spiked Samples for Sulphonamides on Charm II Assay

Sample Number	Control Point	Average CPM (n=2)	Results
1	790	600	positive
2	790	623	positive
3	790	572.5	positive
4	790	562,5	positive
5	790	569	positive
6	790	537.5	positive
7	790	600	positive
8	790	608	positive
9	790	422	positive
10	790	552.5	positive

None of the blank and spiked samples gave any false results. These sample results were confirmed by HPLC. For calibration curves 100, 200, 300 and 400 ppb mix standards were used. Calibration curves are shown on Appendix E.

Table 5.16. Goodness of Fit for Sulphonamide Calibration

Sulphonamides	R^2
Sulfamethazine	0.99915
Sulfadimethoxine	0.99920
Sulfamerazine	0.99927
Sulfathiazole	0.99929
Sulfamethoxazole	0.99933
Sulfanilamide	0.99940
Sulfadiazine	0.99934

A good linearity of the method was observed with a correlation coefficient ranging from 0.99915 to 0.99940 as shown in Table 5.16. The method showed good linearity in the concentration range from 100 ppb to 400 ppb for the sulphonamide antibiotics in milk.

Blank samples and spiked samples screened by Charm II Assay were reconfirmed by HPLC. Table 5.17 and Figure 5.3 show results of a blank samples that were analysed by HPLC. Whereas retention times and chromotogram for sulphonamides are shown in Table 5.18 and Figure 5.4 .

Table 5.17. Concentration of Sulphonamide Antibiotics in Blank Sample

RetTime [min]	k'	Sig	Amount [ng/ul]	Symm.	Width [min]	Plates	Signal /Noise	Name
5.954	-	1	not f	0.00	-	-	-	S.AMÝDE
10.879	-	1	not f	0.00	-	-	-	S.DÝAZÝNE
12.491	-	1	not f	0.00	-	-	-	S.TÝAZOLE
14.169	-	1	not f	0.00	-	-	-	S.MERAZÝNE
17.413	-	1	not f	0.00	-	-	-	S.METAZÝNE
22.826	-	1	not f	0.00	-	-	-	S.METAKSOLE
28.892	-	1	not f	0.00	-	-	-	S.DÝMETOKSÝN

Table 5.18. Concentration of Sulphonamide Antibiotics in Spiked Sample

RetTime [min]	k'	Sig	Amount [ng/ul]	Symm.	Width [min]	Plates	Signal /Noise	Name
5.989	-	1	137.18408	0.91	0.2808	2520	289.7	S.AMÝDE
10.980	-	1	135.76590	0.94	0.3024	7304	522.5	S.DÝAZÝNE
12.619	-	1	134.53890	0.92	0.3024	9646	4.9	S.TÝAZOLE
14.281	-	1	134.01600	0.95	0.3132	11519	10.0	S.MERAZÝNE
17.518	-	1	127.83052	0.98	0.3132	17331	259.0	S.METAZÝNE
22.937	-	1	135.71974	1.01	0.3360	25817	56.3	S.METAKSOLE
28.958	-	1	131.93404	0.95	0.3132	47361	128.1	S.DÝMETOKSÝN

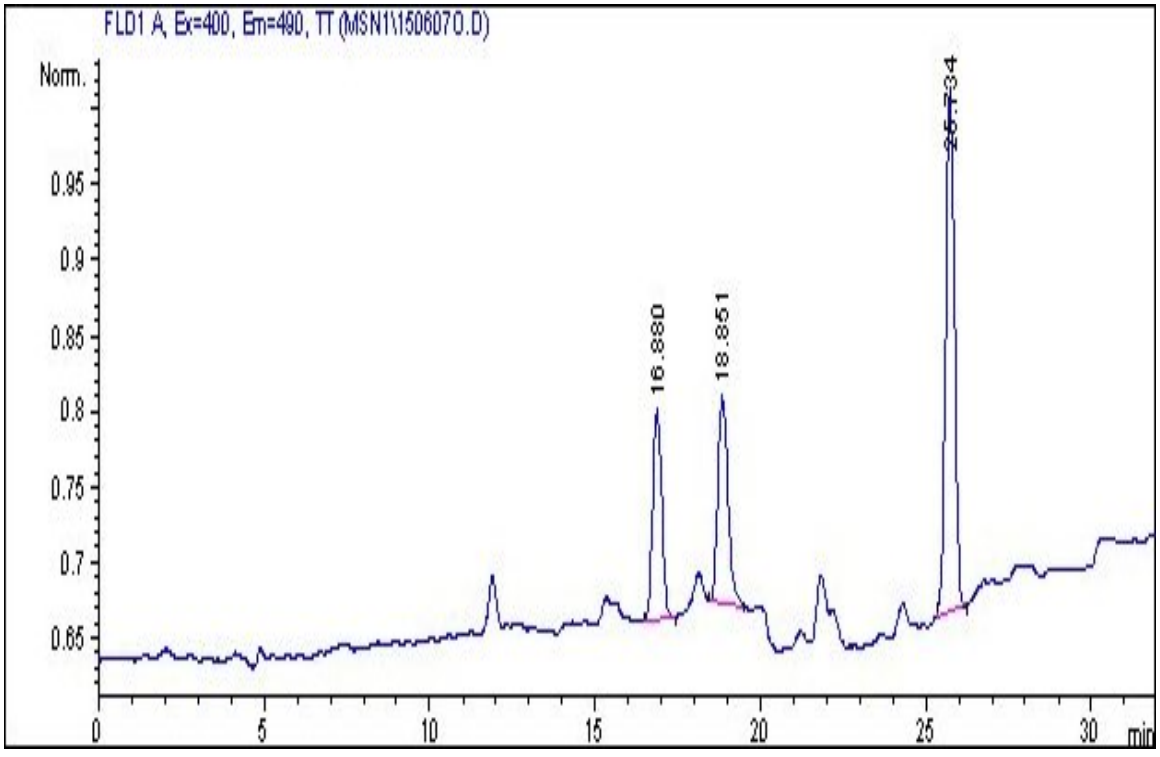


Figure 5.3. Chromatogram Obtained for Sulphonamides by HPLC from Blank Sample

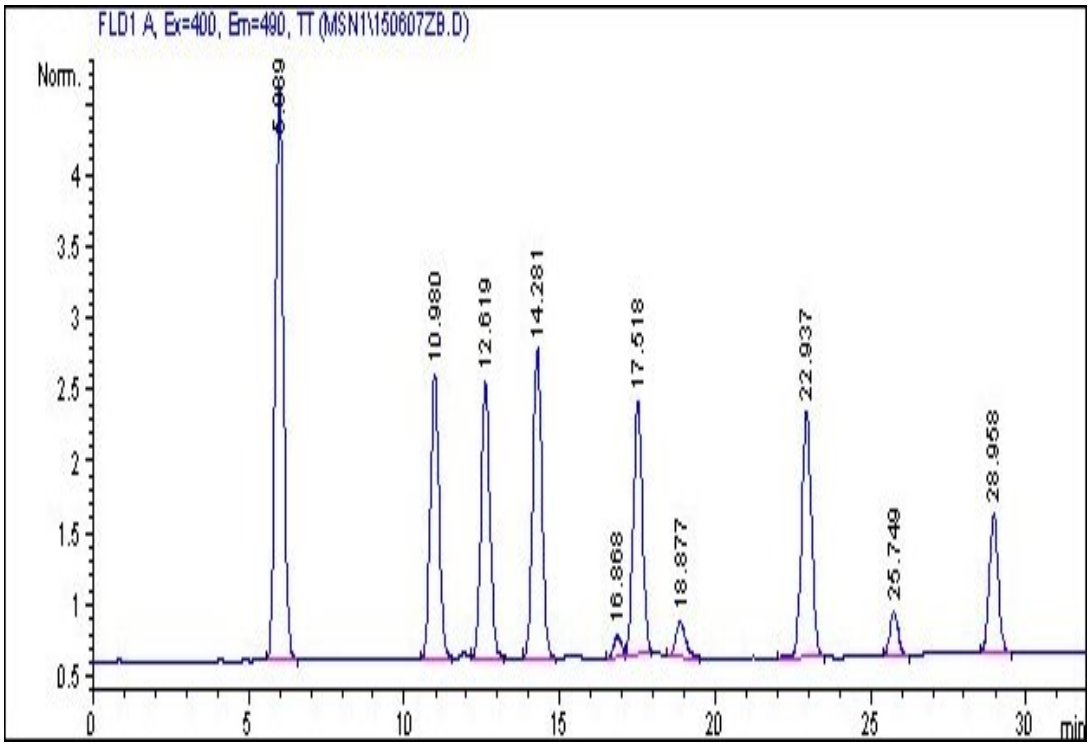


Figure 5.4. Chromatogram Obtained for Sulphonamides by HPLC from Spiked Sample

Figure 5.3 and 5.4 show examples of typical HPLC traces of standards of a blank and a spiked (100 ppb of each drug) milk sample obtained under the established procedure. The method gave good results for detection and identification of sulphonamides without interfering compounds in the resulting extract. The analysis of one sample was accomplished within 30 minutes.

It was confirmed that Charm II Assay didnt give any false results for determination of sulphonamide antibiotics. Average recoveries, Standard deviations and relative standard deviations for 10 spiked samples were given in Table 5.19.

Table 5.19. Average, Standart Deviation and Relative Standard Deviation of Recovery Values of Spiked Samples for Sulphonamides

Antibiotic	Average Recovery (%)	SD (%)	RSD (%)
S.methazine	50.4	1.43	2.8
S.diazine	53.4	1.28	2.4
S.merazine	52.7	1.36	2.6

(cont. on next page)

Table 5.19. Average, Standart Deviation and Relative Standard Deviation of Recovery Values of Spiked Samples for Sulphonamides (cont.)

S.methoxazole	52.9	1.31	2.5
S.thiozole	52.7	1.36	2.6
S.dimethoxine	53.1	1.27	2.4
Sulfanilamide	54.6	3.84	7

Smedley (1994) presented a similar method for determination of multiple sulfonamide residues in bovine milk. Chloroform-acetone solution were used for sample extraction. The organic phase was evaporated, dissolved in potassium phosphate solution. It was added n-hexane to remove fatty residues. The aqueous layer was collected and injected to HPLC system. Residues were detected by UV absorption at 265 nm. Two different mobile phases (12% methanol and 30% methanol) were used to determine 8 different sulfonamides. The average recoveries ranged from 56.2% for sulfaquinoxaline to 82.7% for sulfamethazine in the 12% methanol mobile phase. RSD ranged from 5.7% for sulfaquinoxaline to 10.8% for sulfamethazine. The recoveries were higher compared to our study. In our study mobile phase was 1% acetic acid and wavelenght was used between 400-495 during HPLC analysis. Different mobile phases and wavelenght might be one the reasons accounting different results for recoveries.

5.3.3. Confirmation of Tetracycline Antibiotics in Milk

For confirmation of tetracycline residues, a total of 20 blank samples and 20 spiked samples including the dublicates (100 ppb for each tetracycline standards) were tested with Charm II Assay and reconfirmed by HPLC. Table 5.20 and Table 5.21 present the results for blank and fortified samples (100 ppb mix for each compound of tetracycline).

Table 5.20. Results of Blank Samples for Tetracyclines on Charm II Assay

Sample Number	Control Point	Average CPM (n=2)	Results
1	1229	1551	Not found
2	1229	1352	Not found
3	1229	1266	Not found

(cont. on next page)

Table 5.20. Results of Blank Samples for Tetracyclines on Charm II Assay (cont.)

4	1229	1735	Not found
5	1229	1454	Not found
6	1229	1358	Not found
7	1229	1327	Not found
8	1229	1850	Not found
9	1229	1351	Not found
10	1229	1846	Not found

Table 5.21. Results of Spiked Samples for Tetracyclines on Charm II Assay

Sample Number	Control Point	Average CPM (n=2)	Results
1	1229	650.5	positive
2	1229	668.5	positive
3	1229	830	positive
4	1229	822.5	positive
5	1229	662.5	positive
6	1229	674	positive
7	1229	765	positive
8	1229	775.5	positive
9	1229	968	positive
10	1229	846	positive

None of the blank and spiked samples gave any false results. For calibration curves used in HPLC analysis 50, 100, 150, 200 and 250 ppb mix standards were used. Calibration curves for tetracyclines are shown in Appendix F. The lowest correlation coefficent was 0.99880 for doxycycline as it can be seen in Table 5.22

Table 5.22. Goodness of Fit for Tetracycline Calibration

Tetracyclines	**R^2**
Tetracycline	0.99988
Chlortetracycline	0.99933
Oxytetracycline	0.99947
Doxycycline	0.99880

Blank samples and spiked samples that were screened by Charm II Assay were confirmed by HPLC. Representative chromatograms of a blank and of a spiked sample with 100 ppb of tetracyclines are shown in Figure 5.5 and 5.6, respectively.

Quantification was carried out by comparison of the analyte peak areas versus calibration curve.

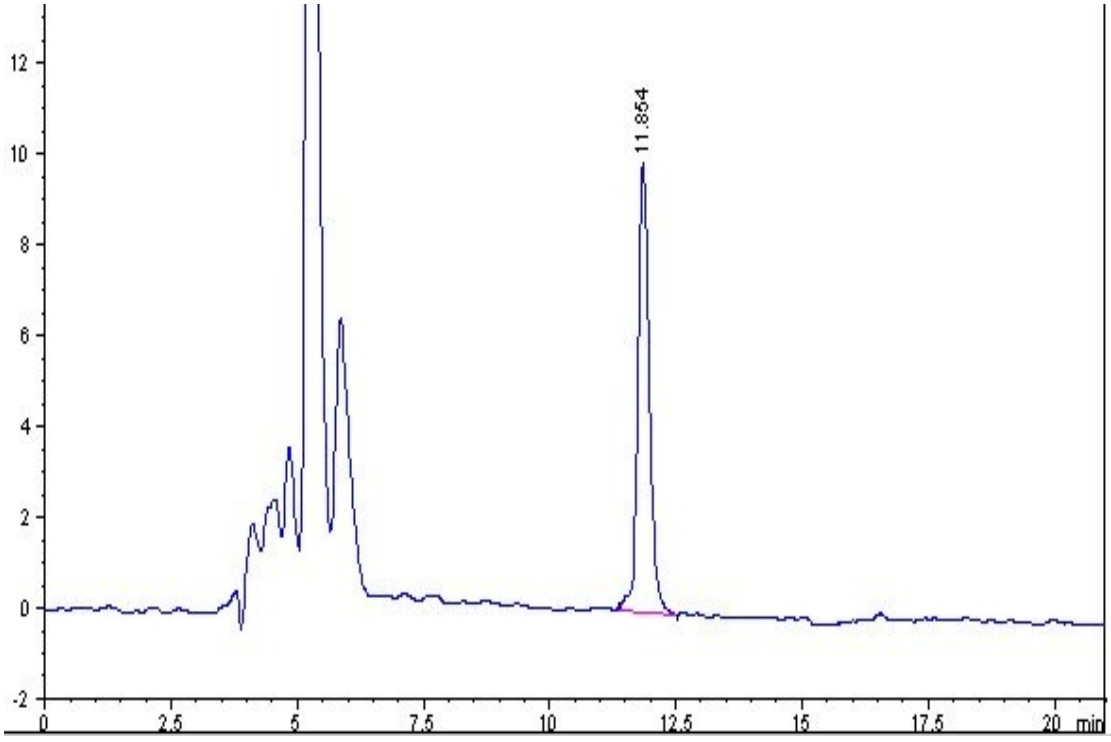


Figure 5.5. Chromatogram of a Blank Sample for Tetracyclines

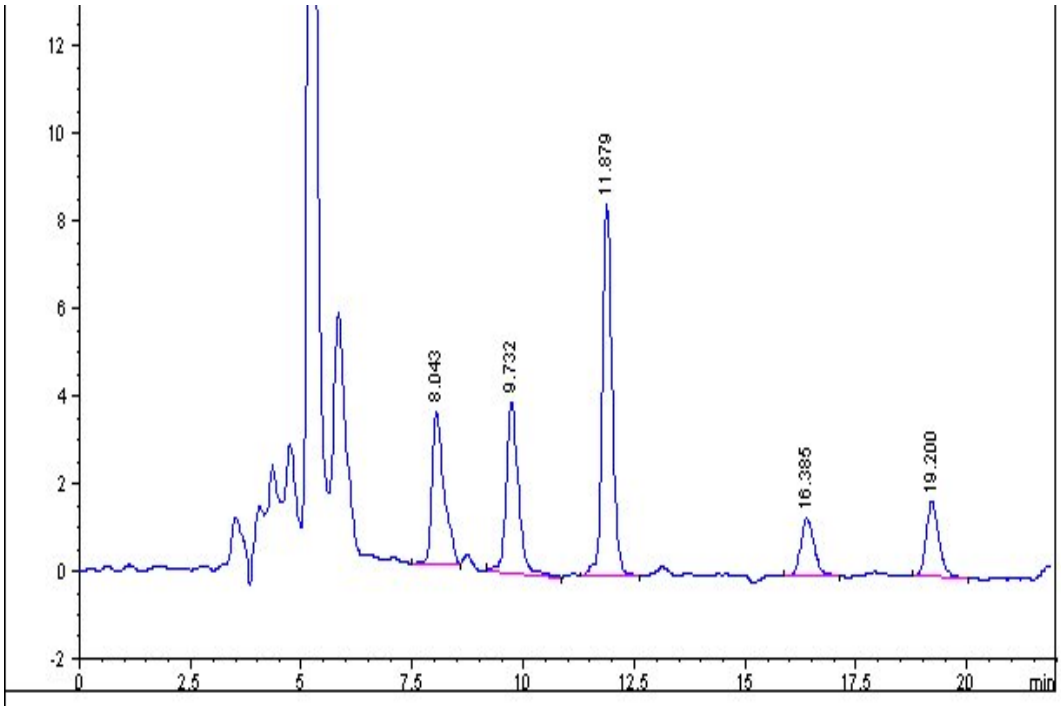


Figure 5.6. Chromatogram of a Spiked Sample for Tetracyclines

No interferences were observed other than interested compounds that were analysed. Table 5.23 shows a HPLC report for a blank sample. Also, detail report for a spiked sample is shown in Table 5.24.

Table 5.23. Concentration of Concentration of Tetracycline Antibiotics in Blank Sample

RetTime [min]	Sig	Type	Area [mAU*s]	Amt/Area	Amount [ng/ul]	Grp	Name
8.237	1		-	-	-		otc
9.988	1		-	-	-		tc
16.752	1		-	-	-		ctc
19.536	1		-	-	-		dc
Totals :					0.00000		

Table 5.24. Concentration of Concentration of Tetracycline Antibiotics in Spiked Sample

RetTime [min]	Sig	Type	Area [mAU*s]	Amt/Area	Amount [ng/ul]	Grp	Name
8.043	1	BB	65.45097	2.64481	173.10536		otc
9.732	1	BBA	74.35610	1.91891	142.68256		tc
16.385	1	PBA	28.05911	4.14482	116.29996		ctc
19.200	1	BBA	34.85766	3.93981	136.98404		dc
Totals :					569.07191		

It was confirmed that Charm II Assay did not give any false results for the determination of tetracycline antibiotics. Average of recoveries, SD (Standard deviation) and RSD (relative standard deviation) results for 10 spiked samples were given in Table 5.25.

Table 5.25. Average, Standard Deviation and Relative Standard Deviation of Recovery Values of Spiked Samples for Tetracyclines

	Tetracycline	Chlorotetracycline	Oxytetracycline	Doxycycline
Average recovery (%)	68.0	61.5	84.8	64.9
Standard deviation (%)	2.16	6.35	3.51	6.60
RSD (%)	3.19	10.3	4.1	10.7

Cinquina et al. (2003) developed a method for oxytetracycline, tetracycline, chlorotetracycline and doxycycline determination in bovine milk by HPLC. Milk samples were homogenised and extracted with 20% trichloracetic acid and McIlvaine buffer. It was centrifuged and purified with SPE HLB cartridge. The analysis were performed using the mobile phase of 0.01 M oxalic acid-acetonitrile-methanol on C8 column at 365 nm. The average recoveries ranged from 83.5% to 91.9% for 100 ppb spiked samples. Our method was a modified model of this study. Mobile phase and solid phase extraction differed from this method. Recoveries was affected negatively by these differences .

Another method were presented by Moats and Harik-Khan (1995) for determination of tetracycline, oxytetracycline and chlorotetracycline. Milk (5 ml) was extracted and deproteinized with 1 ml of 1 N HCl and 15 ml of acetonitrile. The water layer after adding hexane and methylene chloride was evaporated. It was filtered and analized by PLRP-S column with a mobile phase of 0.02 M H_3P0_4 and 0.01 M sodium decanesulfonate-acetonitrile. Recoveries were greater than 80% which were above our results.

5.4. Real Sample Analysis

The developed HPLC method was adopted for the confirmatory analysis of milk samples that were collected by Bornova Veterinary Research and Control Institute. The samples were analysed with Charm II Assay test first and presumptive positive result were analysed by HPLC. 81 samples were screened by Charm II Assay for beta-lactam antibiotics and 9 of them were presumptive positive. These positive samples were then confirmed by HPLC. Six of them were found negative by HPLC. Respectively 6.5 ppb penicilin-G, 23.8 ppb ampicillin-19.9 ppb oxacillin and 24 ppb oxacillin was found in three samples. MRL level for penicillin and ampicillin set by FDA (Food and Drug Administration) is 4 ppb for each. 2 out of 81 samples presented violative values of ampicillin and penicillin-G.

Among 44 milk samples assayed for sulphonamide, 15 samples were determined as positive with Charm II test kit. After HPLC confirmation 12 of them were found as negative and three of them contained 40 ppb sulfadiazine, 119 ppb sulfamethazine and 87.6 ppb sulfadimethoxine, respectively. Only one sample presented violative level of sulphonamide.

46 samples were analysed for tetracycline residues and 9 out of 46 samples were found positive with Charm II Assay. Four of them were found positive after confirmation by HPLC. In one sample 24.3 ppb oxytetracycline, in another sample both 22.1 ppb oxytetracycline and 58.5 ppb chlorotetracycline were found. Similarly 59.1 ppb tetracycline and 46 ppb oxytetracycline were found in another two samples. But all of them were under MRL levels.

Table 5.26 shows the summary of evaluation of positive raw milk samples tested between 2006 and 2007. Violative levels of antibiotics were found in 2 confirmed positive samples for beta-lactams. For sulphonamides only one samples were found above MRL level. Tetracycline analysis did not detect any level that was above MRL. This part of the study was conducted in order to present an overview on the antibiotic residues in real milk samples collected from different parts of Turkey. Because of confidentiality the origin of region could not be outlined.

Table 5.26. Summary of Tested Milk Samples

Group of Antibiotic	Total samples	Screened Positive	Confirmed Positive	Violative level
Beta-lactams	81	9	3	2
Sulphonamide	46	15	3	1
Tetracyclines	44	9	4	-

As a summary, 11.1% of β-lactam samples were screened positive and 3.7% of the samples were confirmed positive. The percentage of samples showed violative levels was found 2.45%. For sulphonamides approximately 33% of samples tested positive but only 6.5% of samples were confirmed positive by HPLC. 2.1% of samples showed violative levels for sulphonamide residues. No violative levels was observed for tetracycline residues. 20.5% of samples were screened positive and only 9.1% of them were confirmed positive by HPLC.

5.5. Commercial Milk Analysis

Five different commercial milk samples were supplied from local supermarket and analysed for antibiotic existance. First, they were assayed by Charm II Assay and

then presumptive positive results were confirmed by HPLC. Results on Charm II counter are shown in Table 5.27, 5.28 and 5.29.

Table 5.27. Charm II Assay Results for Beta-lactam Group of Antibiotics

Samples	Control Point	Average of CPM	Result
Brand 1	864	1095	negative
Brand 2	864	947.5	negative
Brand 3	864	968,5	negative
Brand 4	864	1071.5	negative
Brand 5	864	936	negative

Table 5.28. Charm II Assay Results for Sulphonamide Group of Antibiotics

Samples	Control Point	Average of CPM	Result
Brand 1	790	707.5	positive
Brand 2	790	874	negative
Brand 3	790	602.5	positive
Brand 4	790	762.5	positive
Brand 5	790	685.5	positive

Table 5.29. Charm II Assay Results for Tetracycline Group of Antibiotics

Samples	Control Point	Average of CPM	Result
Brand 1	864	1686	negative
Brand 2	864	1330	negative
Brand 3	864	968.5	negative
Brand 4	864	1476.5	negative
Brand 5	864	1888	negative

All samples were confirmed by HPLC after Charm counting. Table 5.30 shows confirmation results for beta-lactam residues. All samples were free of beta-lactam antibiotics.

Table 5.31 shows the results of HPLC by comparing Charm II Assay results for sulphonamides. 4 out of 5 samples was counted as positive by Charm II Assay but after confirmation by HPLC it was seen that they were negative samples for the sulphonamide groups that was investigated. Charm II Assay can detect 16 different types of sulphonamide group of residues. Because of that it was concluded that there might have existed some other sulphonamide group residues in those milk samples that HPLC could not detect, since the method used only included 7 compounds of this group. No positive results for tetracycline were observed as shown in Table 5.32

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Table 5.30. HPLC and Charm II Assay Results for Beta-lactams

Samples	Charm Results	HPLC Results
Brand 1	-	-
Brand 2	-	-
Brand 3	-	-
Brand 4	-	-
Brand 5	-	-

Table 5.31. HPLC and Charm II Assay Results for Sulphonamides

Samples	Charm Results	HPLC Results
Brand 1	+	-
Brand 2	-	-
Brand 3	+	-
Brand 4	+	-
Brand 5	+	-

Table 5.32. HPLC and Charm II Assay Results for Tetracyclines

Samples	Charm Results	HPLC Results
Brand 1	-	-
Brand 2	-	-
Brand 3	-	-
Brand 4	-	-
Brand 5	-	-

As it can be seen no positive results were observed for tetracyclines residues in milk also. The experiments on commercial milks showed that they have good quality and can be considered save for consumer health.

At the Charm II test, false-positive sample results were found which is tolerable for a screening test that has to be very sensitive, but not very selective. The most important thing for screening tests are that they can not give false-negative results. Because first step for confirmation of antibiotics usually consists of a rapid test and only presumptive positive results are analysed by HPLC. False positive results might be observed if the milk sample is waited long before analysis.

Somatic cell count (SSC), lactoferrin, lysozyme, and other products of inflammation can also cause false positive results in milk. When the concentration of SSC increases, false positive results also increase (Contreras, et al. 1997).

CHAPTER 6

CONCLUSION

Charm II assay antibotic residue test were succesful at screening antibiotic residues in milk.Validation of antibiotics were done to prove that the screening method is suitable to detect MRL levels of antibiotic residues that have been investigated. No accuracy rate were estimated below %95. The test never gave any false negative results with samples that were added with mix standards of antibiotic groups during confirmation study. The reason that it gave some positive results during real samples analysis might be the detection levels of antibiotics on HPLC. Charm II test's sensitivity level might be higher than the confirmation method. For quick determination of antibiotics Charm II assay is very reliable, however since it does not give any information about the amount of antibiotic that is found in milk, it needs to be confirmed by another method.

No reports in Turkey are available for confirmation and investigation of antibiotic residues in milk by screening methods. Therefore, this study will provide a guideline for further studies.

The improvement of analytical methods that enable to detect multiresidues in animal-derived food will give a better knowledge appertain to spectrum of antibiotics. Like we found in this study, sometimes there might be two or in one case three residues in the same milk. This may bring a question of establishing MRL's for "total antibiotics" in milk. This approach would lead to improve methods that enable not only the detection of just a few compounds of same class but also be included other classes of antibiotics.

The main focus should be on the elimination of false positive results by a proper confirmation testing with a quantitative assays rather than qualitative assays. Screening and confirmation tests should be used on farm programs for disease prevention, treatments and effective record keeping .

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APPENDIX A

VALIDATION RESULTS FOR BETA-LACTAM RESIDUES

Table A.1. Validation Results for Beta-lactam

Blank Samples	Control Point	CPM	Results
1	596	765	not found
2	596	739	not found
3	596	777	not found
4	596	779	not found
5	596	807	not found
6	596	744	not found
7	596	681	not found
8	596	719	not found
9	596	912	not found
10	596	739	not found
11	596	853	not found
12	596	715	not found
13	596	736	not found
14	596	877	not found
15	596	754	not found
16	596	742	not found
17	596	846	not found
18	596	674	not found
19	596	706	not found
20	596	811	not found

Table A.2. Validation Results for Oxacillin

30 ppb Oxacillin Loaded Samples	Control Point	CPM	Results
1	596	446	positive
2	596	410	positive
3	596	485	positive
4	596	446	positive
5	596	498	positive
6	596	419	positive

(cont.on next page)

Table A.2. Validation Results for Oxacillin (cont.)

7	596	480	positive
8	596	357	positive
9	596	406	positive
10	596	377	positive
11	596	451	positive
12	596	469	positive
13	596	449	positive
14	596	505	positive
15	596	411	positive
16	596	453	positive
17	596	458	positive
18	596	450	positive
19	596	448	positive
20	596	473	positive

Table A.3. Validation Results for Cloxacillin

30 ppb Cloxacillin Loaded Samples	**Control Point**	**CPM**	**Results**
1	596	433	positive
2	596	366	positive
3	596	376	positive
4	596	376	positive
5	596	450	positive
6	596	358	positive
7	596	517	positive
8	596	401	positive
9	596	384	positive
10	596	392	positive
11	596	337	positive
12	596	404	positive
13	596	423	positive
14	596	415	positive
15	596	375	positive
16	596	338	positive
17	596	443	positive
18	596	438	positive
19	596	404	positive
20	596	365	positive

Table A.4. Validation Results for Ampicillin

4 ppb Ampicillin Loaded Samples	Control Point	CPM	Results
1	591	553	positive
2	591	443	positive
3	591	537	positive
4	591	451	positive
5	591	380	positive
6	591	461	positive
7	591	457	positive
8	591	555	positive
9	591	427	positive
10	591	406	positive
11	591	356	positive
12	591	356	positive
13	591	538	positive
14	591	445	positive
15	591	417	positive
16	591	456	positive
17	591	540	positive
18	591	511	positive
19	591	451	positive
20	591	576	positive

Table A.5. Validation Results for Dicloxacillin

30 ppb Dicloxacillin Loaded Samples	Control Point	CPM	Results
1	591	346	positive
2	591	312	positive
3	591	440	positive
4	591	330	positive
5	591	392	positive
6	591	342	positive
7	591	423	positive
8	591	328	positive
9	591	334	positive
10	591	354	positive
11	591	319	positive
12	591	302	positive
13	591	334	positive
14	591	361	positive
15	591	310	positive
16	591	337	positive
17	591	319	positive
18	591	452	positive
19	591	347	positive
20	591	738	Not found

Table A.6. Validation Results for Penicilin-G

4 ppb Penicillin Loaded Samples	Control Point	CPM	Results
1	596	349	positive
2	596	328	positive
3	596	279	positive
4	596	307	positive
5	596	400	positive
6	596	247	positive
7	596	270	positive
8	596	297	positive
9	596	255	positive
10	596	264	positive
11	596	239	positive
12	596	230	positive
13	596	308	positive
14	596	469	positive
15	596	287	positive
16	596	262	positive
17	596	307	positive
18	596	254	positive
19	596	310	positive
20	596	285	positive

APPENDIX B

VALIDATION RESULTS FOR SULPHONAMIDES

Table B.1. Validation Results for Blank Samples

Blank Samples	Control Point	CPM	Result
1	470	740	not found
2	470	1459	not found
3	470	1105	not found
4	470	475	not found
5	470	2216	not found
6	470	630	not found
7	470	479	not found
8	470	527	not found
9	470	1017	not found
10	470	924	not found
11	470	906	not found
12	470	837	not found
13	470	625	not found
14	470	1329	not found
15	470	837	not found
16	470	650	not found
17	470	906	not found
18	470	837	not found
19	470	2231	not found
20	470	1161	not found

Table B.2. Validation Results for Sulfamethazine

100 ppb Sulfamethazine Loaded Samples	Control Point	CPM	Results
1	1144	622	positive
2	1144	612	positive
3	1144	551	positive
4	1144	584	positive
5	1144	621	positive
6	1144	551	positive
7	1144	614	positive
8	1144	546	positive
9	1144	613	positive
10	1144	585	positive
11	1144	666	positive

(cont.on next page)

Table B.2. Validation Results for Sulfamethazine (cont.)

12	1144	600	positive
13	1144	602	positive
14	1144	618	positive
15	1144	620	positive
16	1144	610	positive
17	1144	668	positive
18	1144	636	positive
19	1144	476	positive
20	1144	550	positive

Table B.3. Validation Results for Sulfadiazine

100 ppb Sulfadiazine Loaded Samples	Control Point	CPM	Result
1	971	609	positive
2	971	556	positive
3	971	503	positive
4	971	560	positive
5	971	581	positive
6	971	552	positive
7	971	540	positive
8	971	551	positive
9	971	581	positive
10	971	599	positive
11	971	588	positive
12	971	574	positive
13	971	550	positive
14	971	618	positive
15	971	629	positive
16	971	607	positive
17	971	595	positive
18	971	578	positive
19	971	605	positive
20	971	548	positive

Table B.4. Validation Results for Sulfamerazine

100 ppb Sulfamerazine Loaded Samples	Control Point	CPM	Results
1	971	550	positive
2	971	522	positive
3	971	491	positive
4	971	602	positive
5	971	464	positive
6	971	525	positive

(cont.on next page)

Table B.4. Validation Results for Sulfamerazine (cont.)

7	971	583	positive
8	971	557	positive
9	971	540	positive
10	971	550	positive
11	971	598	positive
12	971	581	positive
13	971	581	positive
14	971	570	positive
15	971	532	positive
16	971	603	positive
17	971	552	positive
18	971	601	positive
19	971	566	positive
20	971	600	positive

Table B.5. Validation Results for Sulfamethoxazole

100 ppb Sulfamethoxazole Loaded Samples	Control Point	CPM	Result
1	1144	635	positive
2	1144	500	positive
3	1144	512	positive
4	1144	576	positive
5	1144	514	positive
6	1144	562	positive
7	1144	461	positive
8	1144	610	positive
9	1144	565	positive
10	1144	600	positive
11	1144	488	positive
12	1144	525	positive
13	1144	637	positive
14	1144	643	positive
15	1144	614	positive
16	1144	574	positive
17	1144	593	positive
18	1144	603	positive
19	1144	560	positive
20	1144	570	positive

Table B.6. Validation Results for Sulfathiozole

100 ppb Sulfathiozole Loaded Samples	Control Point	CPM	Results
1	971	646	positive
2	971	563	positive
3	971	503	positive
4	971	568	positive

(cont.on next page)

Table B.6. Validation Results for Sulfathiozole (cont.)

5	971	545	positive
6	971	575	positive
7	971	580	positive
8	971	581	positive
9	971	533	positive
10	971	600	positive
11	971	567	positive
12	971	557	positive
13	971	531	positive
14	971	531	positive
15	971	550	positive
16	971	667	positive
17	971	585	positive
18	971	605	positive
19	971	575	positive
20	971	595	positive

Table B.7. Validation Results for Sulfamethoxine

100 ppb Sulfadimethoxine Loaded Samples	Control Point	CPM	Results
1	971	535	positive
2	971	551	positive
3	971	531	positive
4	971	621	positive
5	971	544	positive
6	971	544	positive
7	971	570	positive
8	971	579	positive
9	971	563	positive
10	971	640	positive
11	971	502	positive
12	971	554	positive
13	971	646	positive
14	971	626	positive
15	971	594	positive
16	971	515	positive
17	971	587	positive
18	971	606	positive
19	971	573	positive
20	971	914	positive

APPENDIX C

VALIDATION RESULTS FOR TETRACYCLINE RESIDUES

Table C.1. Validation Results for Blank Samples

Blank Samples	Control Point	CPM	Results
1	1686	1820	not found
2	1686	1839	not found
3	1686	1856	not found
4	1686	1988	not found
5	1686	1832	not found
6	1686	1795	not found
7	1686	1830	not found
8	1686	2649	not found
9	1686	1937	not found
10	1686	1718	not found
11	1686	1791	not found
12	1686	2031	not found
13	1188	1485	not found
14	1188	1728	not found
15	1188	2145	not found
16	1188	1509	not found
17	1188	2051	not found
18	1188	1414	not found
19	1188	1204	not found
20	1188	1599	not found

Table C.2. Validation Results for Tetracycline

100 ppb Tetracycline Loaded Samples	Control Point	CPM	Results
1	1686	1367	positive
2	1686	1267	positive
3	1686	1145	positive
4	1686	978	positive
5	1686	1231	positive
6	1686	1128	positive
7	1686	1316	positive
8	1686	1067	positive
9	1686	1087	positive
10	1686	1568	positive

(cont.on next pape)

Table C.2 Validation Results for Tetracycline (cont.)

11	1188	1101	positive
12	1188	1076	positive
13	1188	1025	positive
14	1188	1028	positive
15	1188	844	positive
16	1188	867	positive
17	1188	896	positive
18	1188	975	positive
19	1188	856	positive
20	1188	842	positive

Table C.3. Validation Results for Chlorotetracycline

100 ppb Chlorotetracycline Loaded Samples	**Control Point**	**CPM**	**Results**
1	1686	1208	positive
2	1686	1268	positive
3	1686	1340	positive
4	1686	1254	positive
5	1686	1218	positive
6	1686	1198	positive
7	1686	1246	positive
8	1686	1010	positive
9	1686	1134	positive
10	1686	939	positive
11	1188	874	positive
12	1188	1007	positive
13	1188	881	positive
14	1188	1024	positive
15	1188	893	positive
16	1188	925	positive
17	1188	1345	Not found
18	1188	1066	positive
19	1188	919	positive
20	1188	943	positive

Table C.4. Validaiton Results for Doxycycline

100 ppb Doxycycline Loaded Samples	**Control Point**	**CPM**	**Results**
1	1151	953	positive
2	1151	819	positive
3	1151	726	positive
4	1151	906	positive
5	1151	843	positive
6	1151	760	positive
7	1151	782	positive
8	1151	798	positive

(cont.on next page)

Table C.4. Validaiton Results for Doxycycline (cont.)

9	1151	1159	not found
10	1151	771	positive
11	1151	1007	positive
12	1151	793	positive
13	1151	766	positive
14	1151	895	positive
15	1151	952	positive
16	1151	683	positive
17	1151	809	positive
18	1151	950	positive
19	1151	795	positive
20	1151	843	positive

Table C.5. Validation Results for Oxytetracycline

100 ppb Oxytetracycline Loaded Samples	**Control Point**	**CPM**	**Results**
1	1686	1380	positive
2	1686	1218	positive
3	1686	1130	positive
4	1686	1287	positive
5	1686	1001	positive
6	1686	1300	positive
7	1686	1000	positive
8	1686	1114	positive
9	1686	1283	positive
10	1686	1018	positive
11	1181	1162	positive
12	1181	1055	positive
13	1181	1127	positive
14	1181	970	positive
15	1181	948	positive
16	1181	940	positive
17	1181	1100	positive
18	1181	972	positive
19	1181	1125	positive
20	1181	962	positive

APPENDIX D

CALIBRATION CURVES FOR BETA-LACTAM

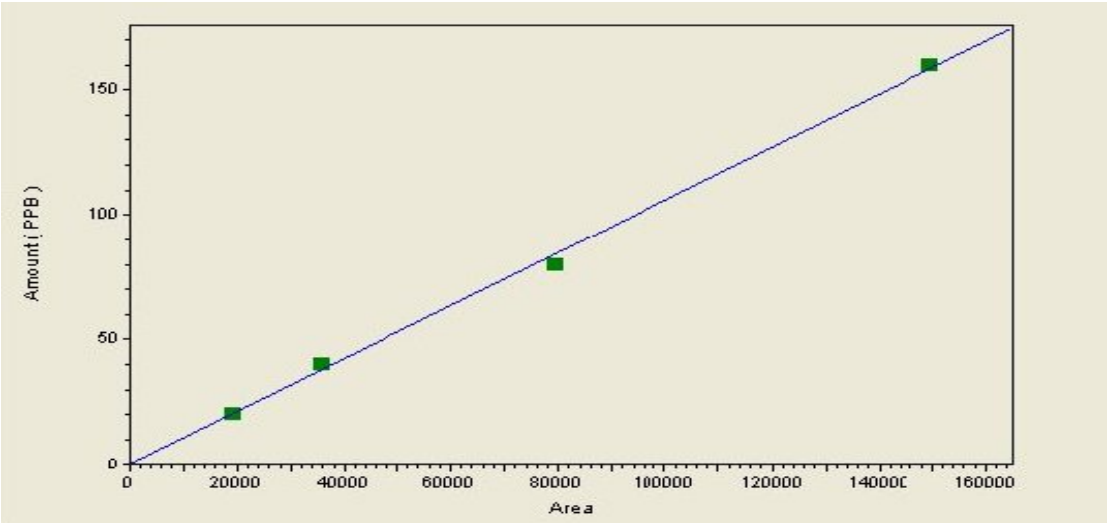

Figure D.1. Calibration Plot for Ampicillin For Concentration Range of 20 ppb-160 ppb

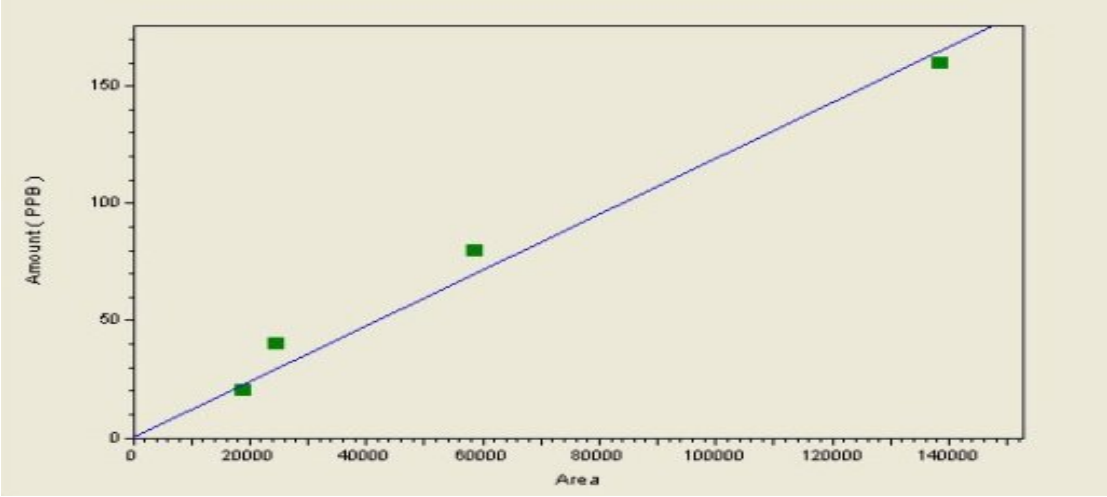

Figure D.2. Calibration Plot for Cloxacillin for Concentration Range of 20 ppb-160 ppb

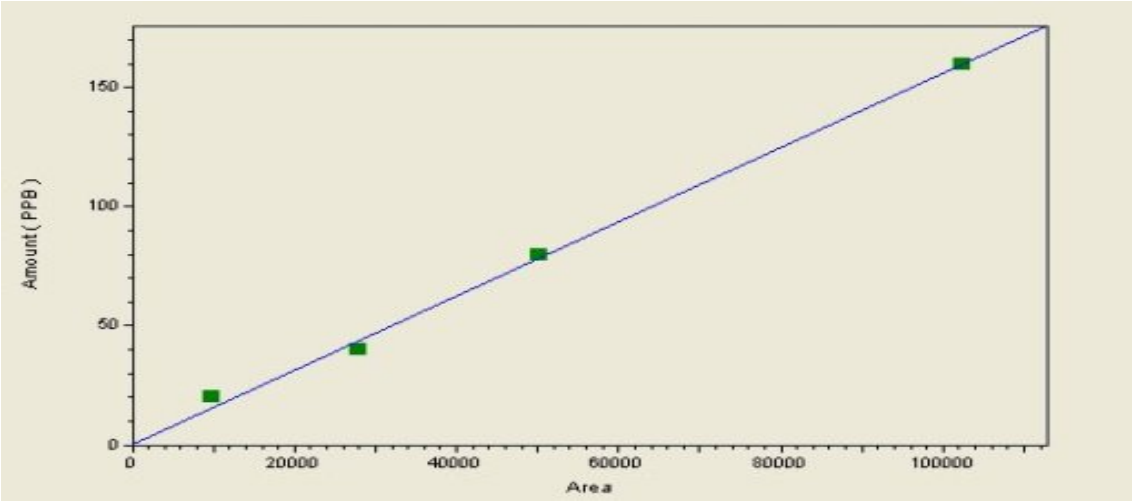

Figure D.3. Calibration Plot for Dicloxacillin for Concentration Range of 20 ppb-160 ppb

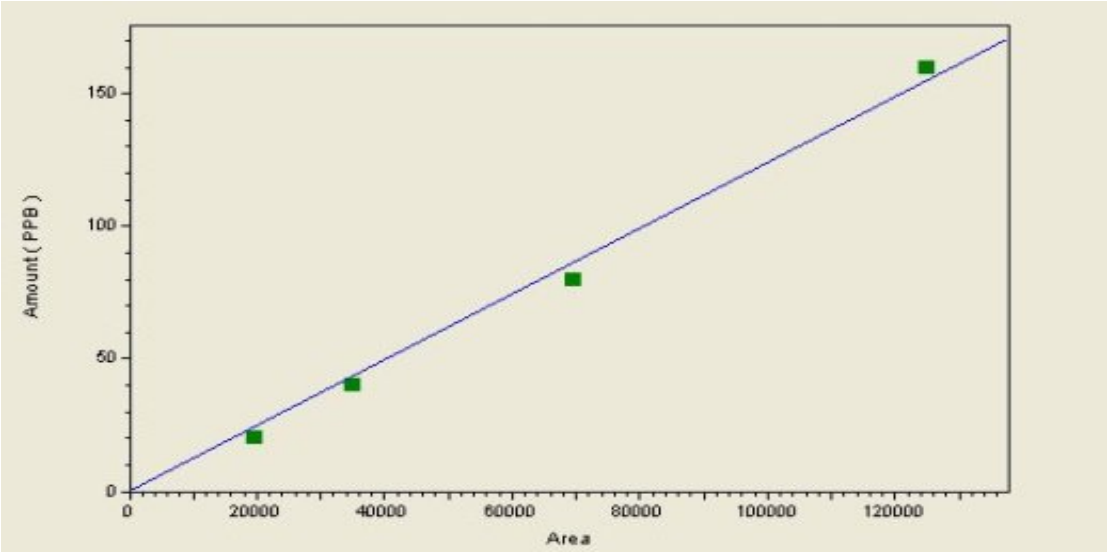

Figure D.4. Calibration Plot for Oxacillin for Concentration Range of 20 ppb-160 ppb

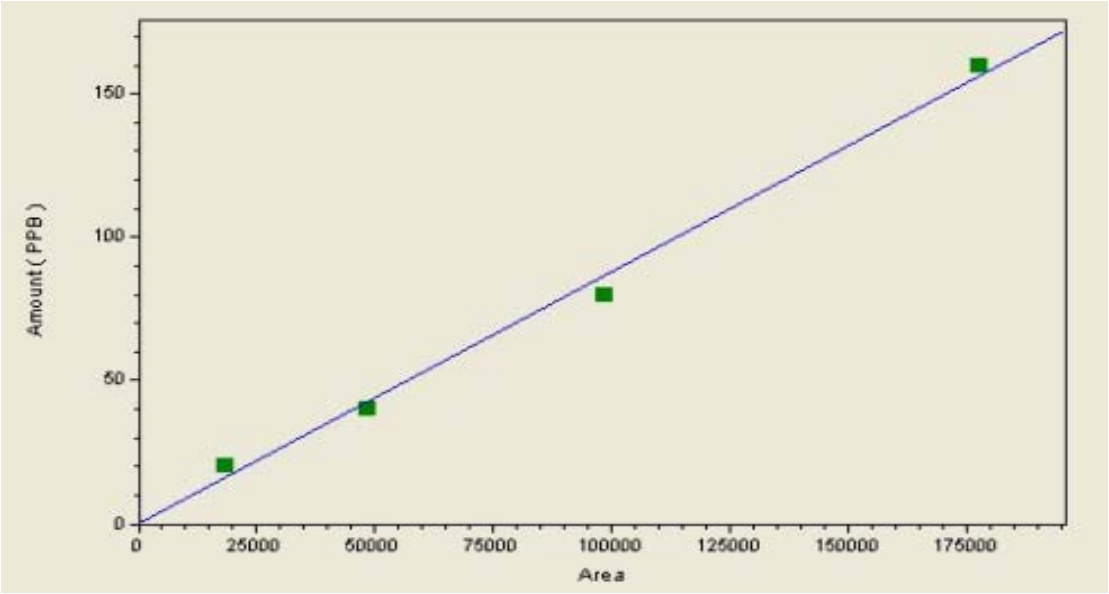

Figure D.5. Calibration Plot for Penicillin-G for Concentration Range of 20 ppb-160 ppb

APPENDIX E

CALIBRATION CURVES FOR SULPHONAMIDES

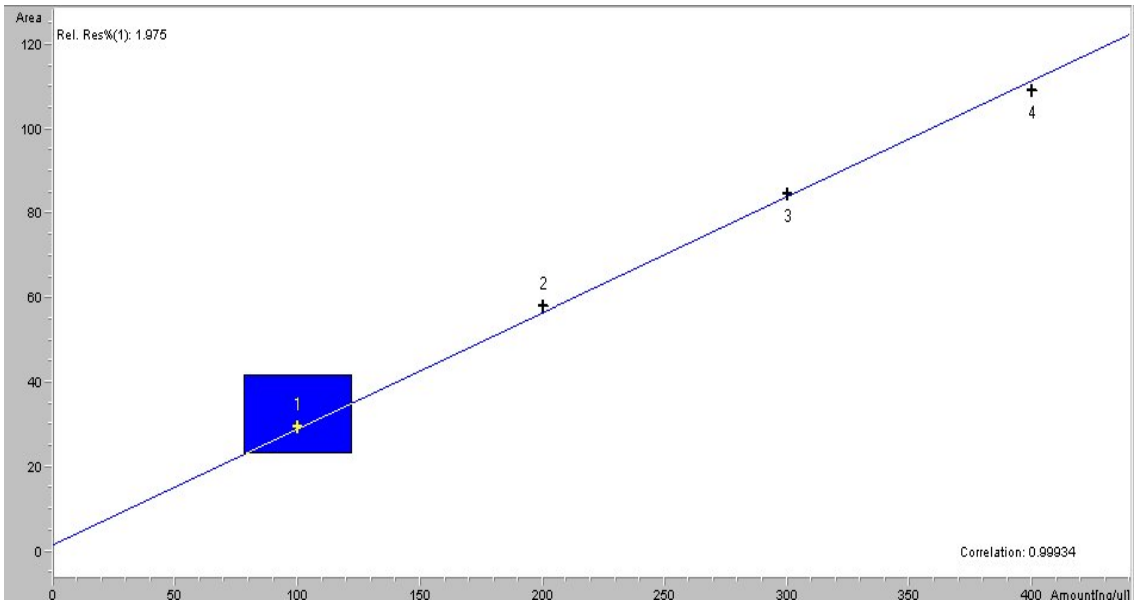

Figure E.1. Sulfadiazine Calibration Curve

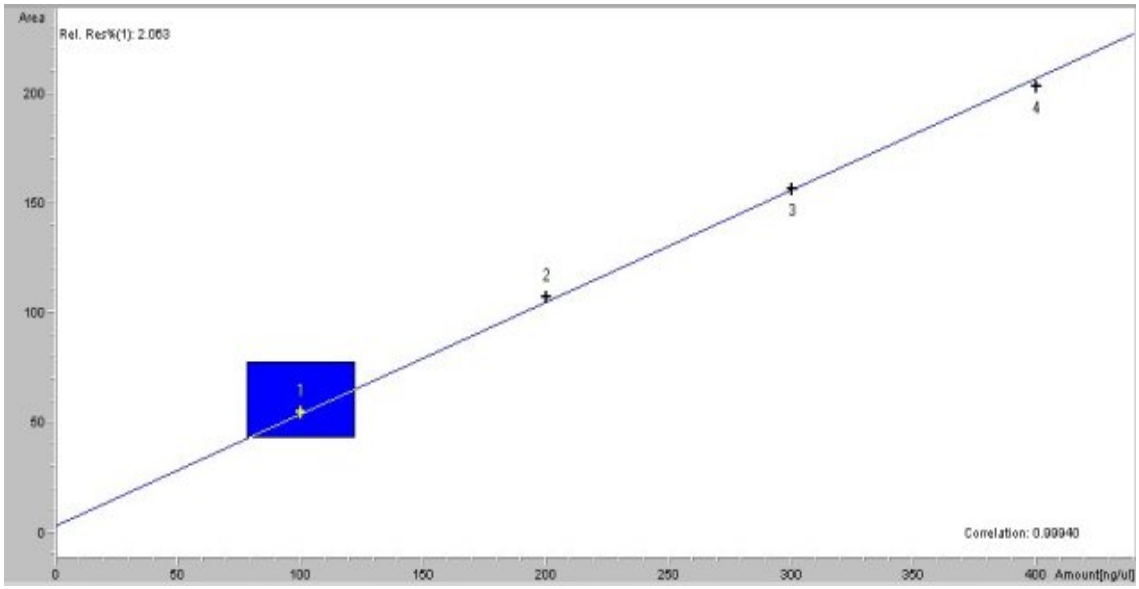

Figure E.2. Sulfanilamide Calibration Curve

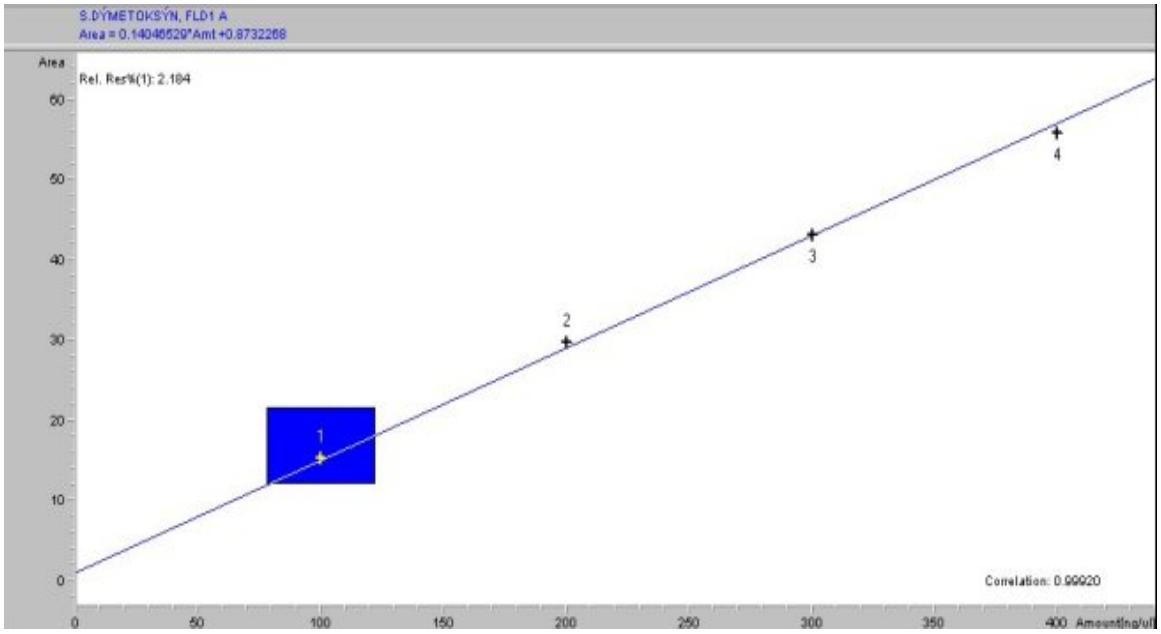

Figure E.3. Sulfadimethoxine Calibration Curve

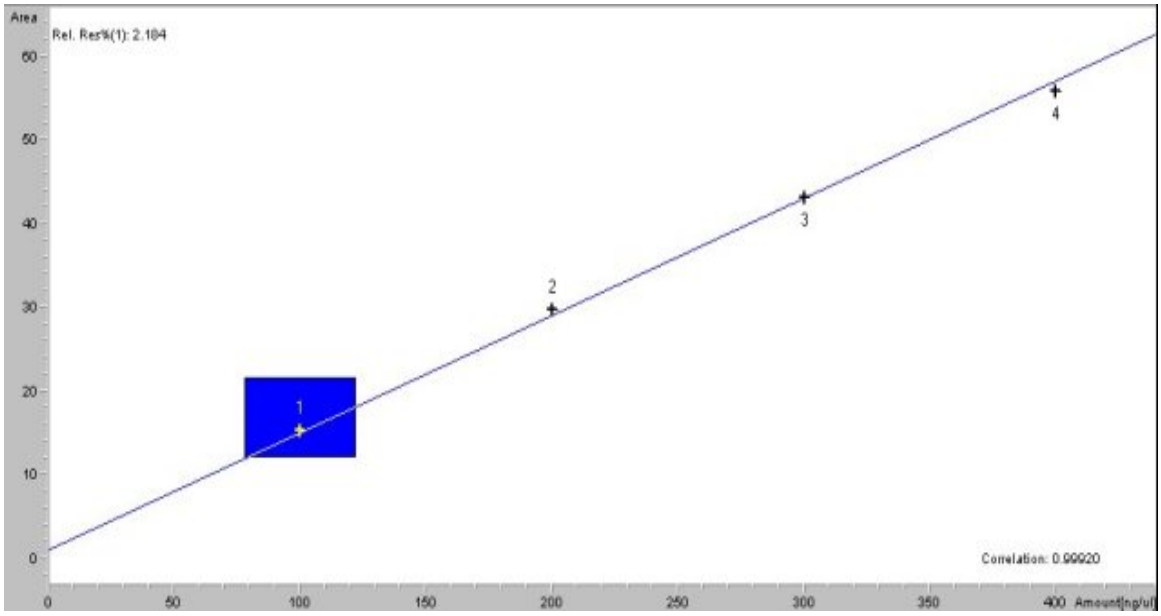

Figure E.4. Sulfamerazine Calibration Curve

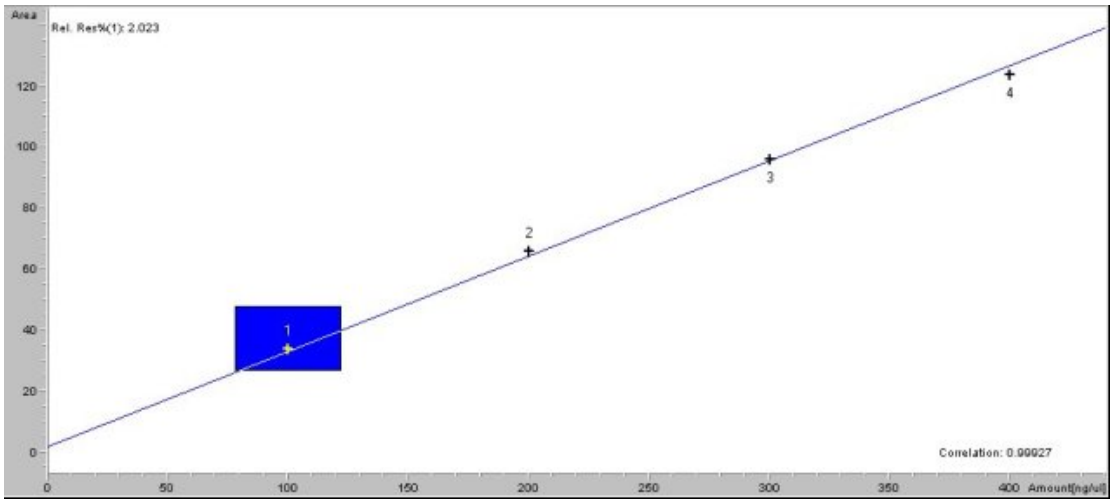

Figure E.5. Sulfamethazine Calibration Curve

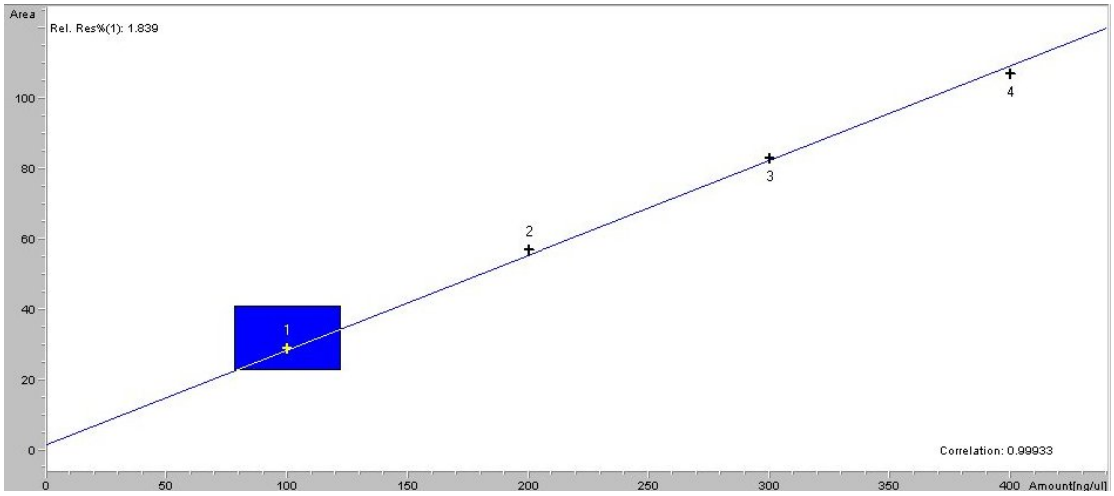

Figure E.6. Sulfamethoxazole Calibration Curve

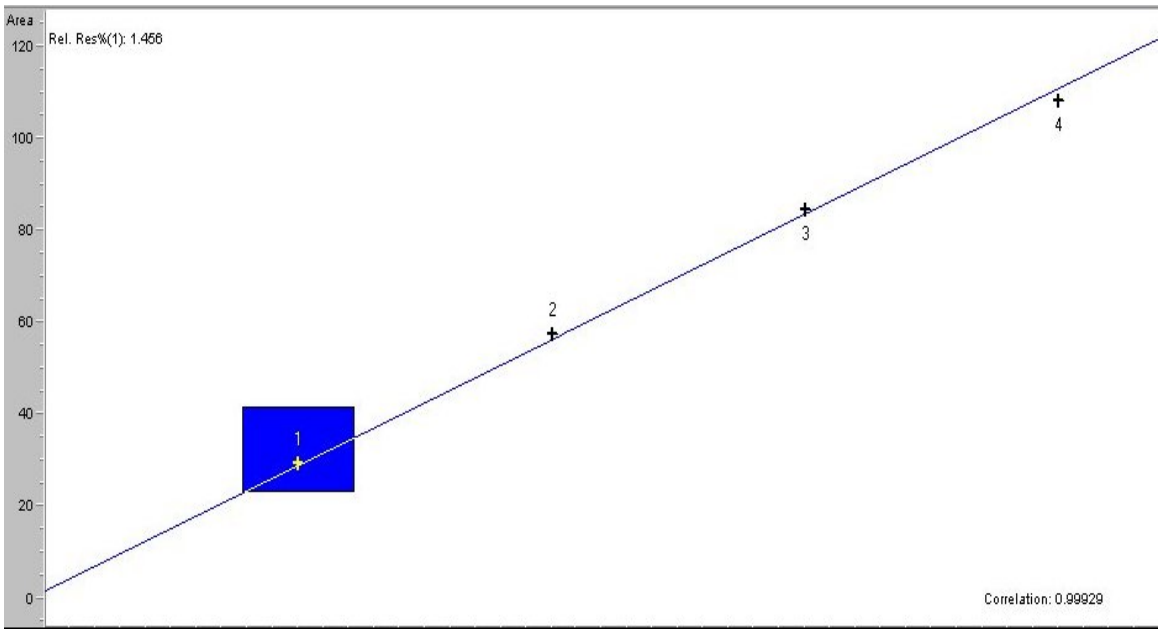

Figure E.7. Sulfathiazole Calibration Curve

APPENDIX F

CALIBRATION CURVES FOR TETRACYCLINES

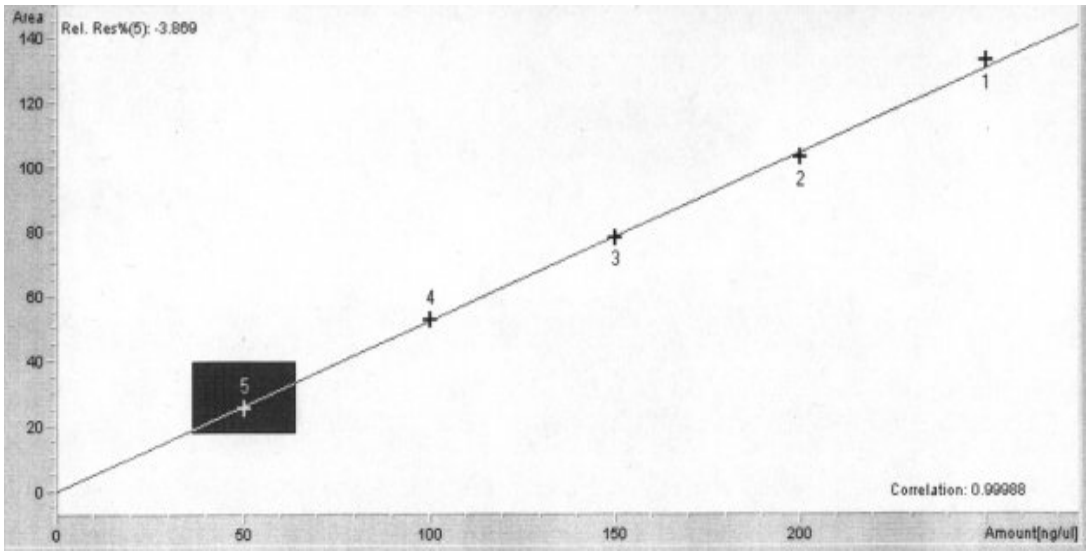

Figure F.1. Tetracycline Calibration Curve

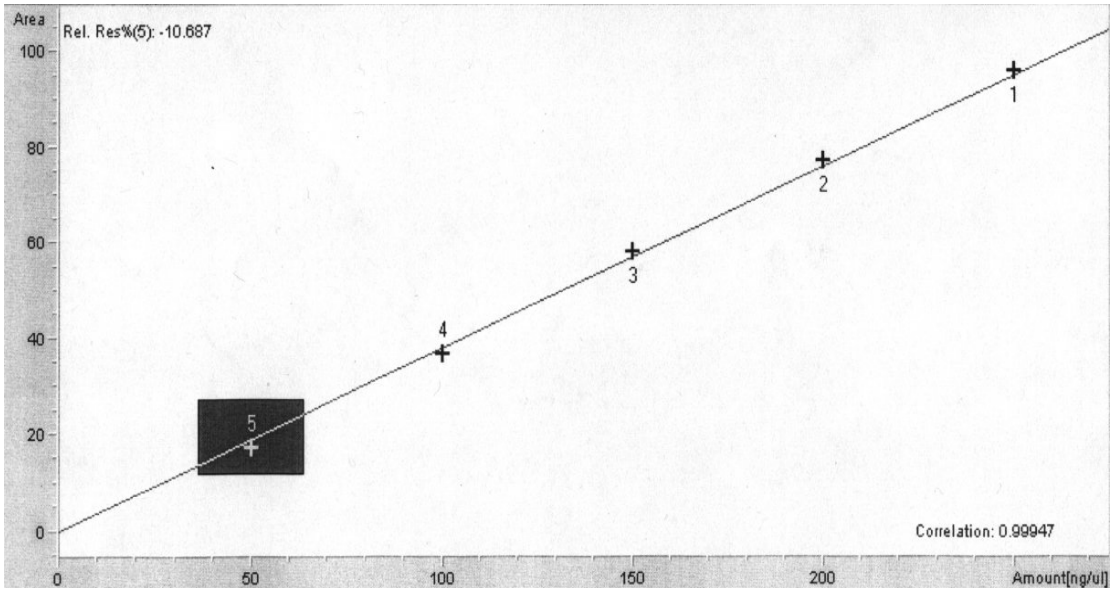

Figure F.2. Oxytetracycline Calibration Curve

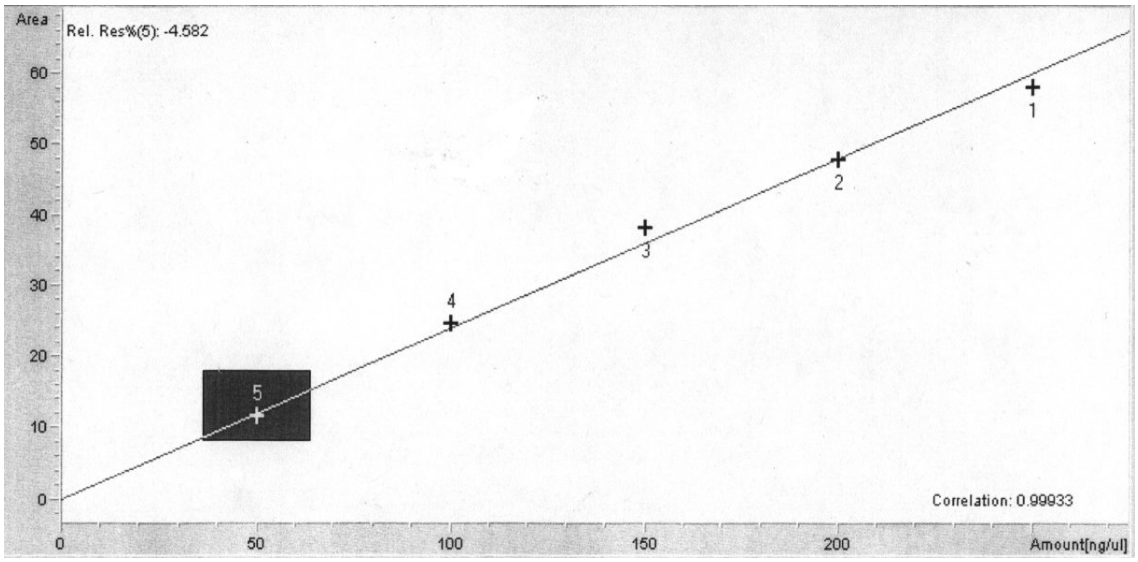

Figure F.3. Chlorotetracycline Calibration Curve

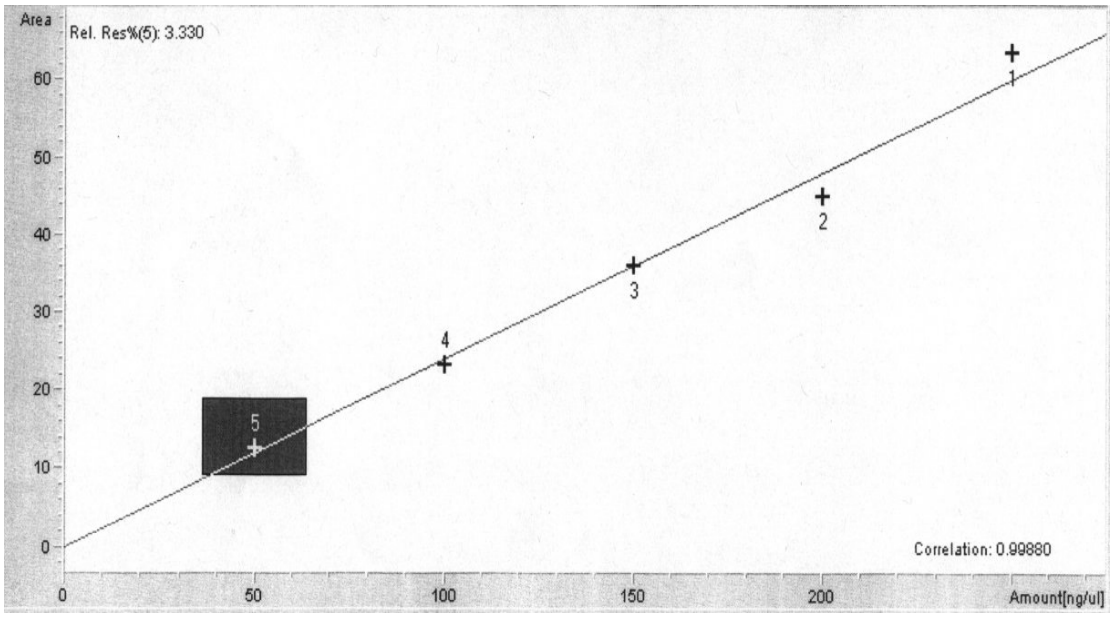

Figure F.4. Doxycycline Calibration Curve