# EFFECT OF PROCESSING PARAMETERS ON THE DETECTION OF ANIMAL DRUG RESIDUES IN MILK

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(ABSTRACT)

The advent of new methods to detect animal drug residues has resulted in a need to independently validate them. The effects of processing milk on the performance of these methods was evaluated. Antibiotic free milk samples were spiked with sulfamethazine, penicillin G, and chlortetracycline at levels of 10, 10 and 30 ppb, respectively. Spiked milk and negative control milk was heat treated, homogenized or heat treated and homogenized. The procedures evaluated for penicillin detection were *Bacillus stearothermophilus* disk assay, a HPLC described by Barker et al., Charm II microbial receptor assay, and CITE Probe and LacTek enzyme immunoassays. The procedures evaluated for sulfonamide detection were an HPLC method described by Long et al., Charm II microbial receptor assay, CITE Probe, LacTek and Signal enzyme immunoassays. The methods evaluated for tetracycline detection were a HPLC method described by Long et al., Charm II microbial receptor assay, and LacTek and CITE Probe enzyme immunoassays. The results indicate that the commercial tests and the disk assay were not adversely affected by processing treatments. Significant treatment differences were found when testing raw Charm II data by analysis of variance but these differences did not effect the overall results of the test. Results of the HPLC method were inconclusive for the three drugs tested.

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# CHAPTER 1

# INTRODUCTION

One definition of antibiotics is "products from living organisms which are not toxic to the producing organisms but are capable, in low concentrations, of inhibiting the growth of one or more microorganisms" (30). This definition does not allow the inclusion of chemically synthesized agents, like sulfonamides, which are classified as chemotherapeutic agents or drugs. For the rest of this thesis, therefore, the term "drugs" will be used to describe both antibiotics and animal drugs. Mechanisms by which drugs act on microorganisms include the inhibition of cell wall synthesis, protein synthesis, nucleic acid synthesis, and the disruption of membrane functions (30,81).

Modern chemotherapy began in the beginning of the century when Ehrlich discovered chemicals that possessed toxicity against cetain microorganisms. He discovered salvarsan, the first chemotherapeutic agent, as a cure for syphilis. Domagk discovered sulfanilamide in 1935 (36). Alexander Fleming discovered penicillin from molds by chance in 1929. Its isolation and development by H. Florey produced the first class of antibiotics and revolutionized medicine (36). Since the time of these early discoveries, many more antibiotics and drugs have been discovered or developed.

A number of these drugs are used in dairy cattle as intramammary administrations in the treatment of mastitis, injections against a variety of diseases, as uterine flushes in the form of boluses following partuition and as prophylactics orally in feed. When drugs are given to lactating dairy cattle, a withdrawal time in

which the milk is withheld from human consumption is usually prescribed. This is to insure that the drug will clear the animal's system and residues from the drug do not get into the milk supply for reasons that will be outlined later.

Residues may be present in milk due to failure to observe the labelled withholding time, using a drug extra-labelly that doesn't have a withholding time prescribed for dairy cattle, accidental transfer of milk from treated cows into drug free milk, prolonged excretion of the drug, and early calving (incomplete withdrawal from dry cow mastitis preparations) (108). One study has shown that management factors that have been found to increase the risk of residues in milk involve frequent use of part time help and use of parlor milking systems (62). The same study showed that risk of residues decreased when farmers used milk residue test kits and when farmers believed that increasing the dose of antibiotic required an increased withholding time.

Concerns about drug residues in milk focus on several issues. From a public health perspective, residues are of concern because of allergen potential, drug resistant pathogens, and toxicity. From an industry prospective, residues are of concern because they can effect milk quality tests and cultured dairy product manufacture.

The drug of greatest concern in inducing an allergic reaction is penicillin. Up to 10% of the general population is estimated to be allergic to penicillin (46,74). Certain people who are highly sensitive to the drug can suffer allergic reactions to very low residue levels in milk. Humans hypersensitive to penicillin have been demonstrated to react to levels as low as 40 $\mu$g (107). There is also the possibility that low doses of penicillin can sensitize a person to penicillin. In animal studies, the best way to sensitize an animal to a drug and get production of antibodies is

through low dose immunization over a period of time. This immunization is usually conducted using parenteral routes. There is very little quantitative data on the dose required to elicit an immune reaction by the oral route (18). There have been cases documented in medical literature of allergic reactions due to the ingestion of penicillin contaminated milk (22,100) The only conclusive documentations of extreme anaphylactic reactions involved with penicillin contaminated food involved contaminated meat products (40,80). About 3.4% of the population is thought to be allergic to sulfonamides so it is of concern as well (11).

There is a concern among some scientists that use of drugs in animal husbandry will select for drug resistance in microorganisms in animals and that this resistance can be passed on to humans (33,50). While it is generally believed that this can happen, some researchers believe that the contribution from animal husbandry is negligible compared to the contrubution from therapeutic use of drugs in humans. The major cause of drug-resistant microorganisms in man is primarily due to the overuse of drugs in human medicine but there is a contribution from agriculture (46).

Another health concern is the toxicity of some drugs. Some drugs that are not approved for use in lactating dairy cattle are used routinely under the FDA's extra-label drug use policy. Sulfonamides and tetracyclines fall into this class. Sulfonamides are suspected of causing thyroid cancer. Tetracyclines can have effects on the developing bones of children and are implicated in renal failure in elderly patients receiving therapeutic levels of the drug (13).

From a dairy product processing perspective, drug residues in milk can cause problems with quality assurance tests and cultured dairy products. Penicillin and oxytetracycline were responsible for causing false positive phosphatase tests (60).

This means that milk could be determined by the test to be inadequately pasteurized even though it was properly pasteurized. Penicillin has also been shown to cause an increase in the methylene blue reduction time (30). Based on this result, milk containing high numbers of microorganisms may be assessed as having a low microbial load. Animal drugs in milk intended for cultured dairy products may cause slow or absent starter growth and acid production, and problems during cheese ripening (61).

In 1988 the Food and Drug Administration (FDA) conducted a survey to detect whether sulfamethazine, a drug illegal for use in dairy cattle, could be found in consumer samples above its safe level of 10 ppb. The drug was found in a disturbingly high number of samples. The FDA and the National Conference on Interstate Milk Shipments (NCIMS), a voluntary federal/state program for the certification of interstate milk shippers, developed a nationwide education program to educate farmers so drug residues could be avoided. The NCIMS met in 1989 and a suggestion that new test methods that could detect drugs that the current regulatory method could not be included into the Pasteurized Milk Ordinance (PMO) was rejected (78). The *B. stearothermophilus* disk assay was the only regulatory method listed for drug residue detection in the PMO, the official milk sanitation code of the United States Public Health Service. In late 1989, the Wall Street Journal and the Center for Science in the Public Interest singled out the shortfalls in drug residue monitoring that was occurring for milk and conducted studies of their own, showing widespread residue contamination. Their concerns were that FDA's extra-label drug use policy allowed farmers and veterinarians to use practically whatever drug they desired and the FDA had no methods to determine if illegal drug residues were present in milk. The *B. stearothermophilus*

disk assay cannot detect anything but ß-lactam antibiotics at adequate levels of detection. Also, adherence to regulations that require new drug sponsors to submit screening methods for the detection of residues of the sponsored drug was not enforced (78). Another concern was incomplete and inadequate information about milk withdrawal times or levels of concern for these extra-label drugs (78,89). An example of problems associated with this is gentamicin. It is only approved for use in swine and has a withdrawal time of 10 to 14 days. The Center for Veterinary Medicine has data documenting it takes more than 70 days to deplete this same drug to the same levels in cattle. If a veterinarian uses the information on the bottle for the withdrawal time in pigs, it's very doubtful that a proper withdrawal time will be prescribed for dairy cattle (89).

These facts resulted in a plethora of media coverage on both sides of the issue and culminated in a congressional subcommittee hearing (12,16,24,26,27,28,29,37, 38,41,42,43,51,71). The FDA responded with surveys of their own showing that there were no confirmed drug residues in milk. These results were released the day before the hearing and were summarily pulled apart by the subcommittee chair, Congressman Ted Weiss of New York. The FDA's own results showed drug residues were found by the Charm II screening method, but when the FDA used its own high-performance liquid chromatography (HPLC) and gas chromatography-mass spectroscopy (GC-mass spec) methods that are more specific but less sensitive, the results could not be confirmed. They then concluded that the Charm II results were false positives.

Because of this extensive media coverage and consumer unrest, the FDA with the NCIMS developed the National Drug Residue Milk Monitoring Program. The program is designed to determine if and what drug residues are present in milk,

determine the extent that farmers, veterinarians and drug distributors comply with regulations, and to provide federal, state and local milk officials with information on educational and enforcement programs (25). The NCIMS also expressed a need for good screening and confirmatory tests (78). Many screening tests were becoming available from industry but had not been comprehensively evaluated. A study was conducted by at Virginia Tech on the ability of these tests to detect the drug residues at levels claimed by the test kit manufacturer. These tests were conducted on spiked raw milk.

Since some state laboratories are reportedly using these screening tests on finished products, and the tests were developed for raw milk, this study was undertaken to determine the ruggedness of the tests to milk processing. Specifically, the study was undertaken to decide whether heat treatment, homogenization, or a combination of both could affect the results of screening methods found to be effective in testing raw milk for drug residues. It is hoped that the facts learned in this study will help settle the public's dissatisfaction with drug residue monitoring programs, and help the dairy farmer and milk processor select valid screening methods so drug residues in milk can be eliminated.

# CHAPTER 2

# LITERATURE REVIEW

## ANIMAL DRUG RESIDUES

### Beta Lactams

This class of antibiotic includes penicillins and cephalosporins. Penicillin was the first clinically useful antibiotic, coming into use in 1941 (85). Benzyl penicillin, also called penicillin G, is produced from *Penicillium*. Procaine penicillin G is one of only three injectable antibiotics approved for lactating dairy cattle (103). The amount and duration of ß-lactam residues in milk are affected by route of administration and number of different antibiotics administered (73). When administered by injection, less than 0.03% of the drug is eluted into the milk. When administered intramammary however, most of the drug is excreted in the milk (107). Penicillin also has the distinction of being the most allergenic antibiotic. Approximately 10% of all individuals who receive penicillin become sensitive to it (46,74). There have also been human cases of allergic dermatitis due to penicillin in milk reported (22,100). Humans hypersensitive to penicillin have been demonstrated to react to as low a dose as 40 $\mu$g of the drug (107).

All penicillins consist of a thiazolidine ring connected to a ß-lactam ring to which a side chain is attached as shown in figure 1 (80,108). The side chain is one factor in deciding the pharmacokinetics of the drug. Another factor is the salts, such as sodium and potassium, with which the penicillins are formulated. Beta-lactams are susceptible to acid hydrolysis of the ß-lactam ring and this destroys its antibiotic activity (85). Katz et al. (46) noted that penicillin residues "survive" all but the most rigorous cooking. Penicillin G is susceptible to the activity of penicillinases that rupture the ß-lactam ring and destroy antibiotic activity. Even though antimicrobial activity is lost, the residues, called penicilloyl residues, may still have allergenic potential, possibly greater potential than that of the parent compound (46). Ruptured penicillins can be found free or bound to serum or tissue proteins. Binding of the parent drug or the penicilloyl metabolite to serum albumin can mask the drug inside the tertiary structure of the albumin and make it inaccessible to antibodies (101,102).

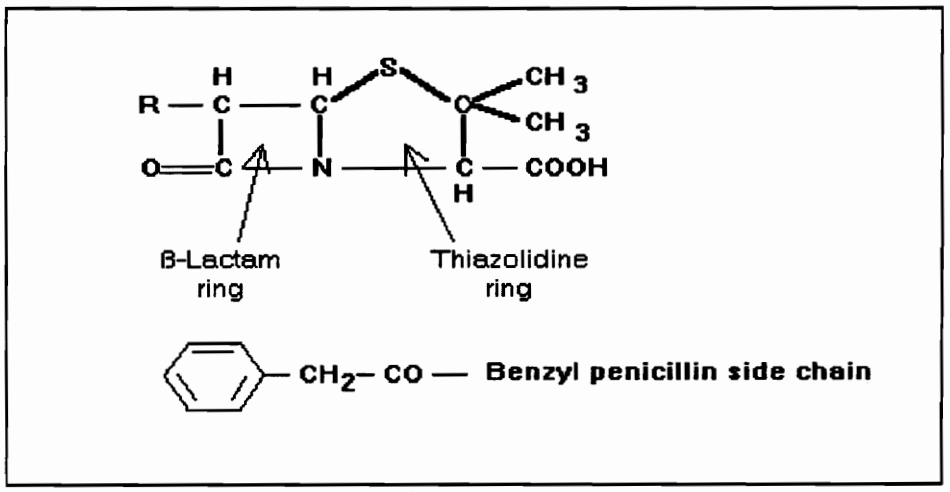


Figure 1. Penicillin structure (80).

Approximately 60% of the penicillin G in a subject can be bound to albumin at one time (17,85). Ionic and hydrophobic bonds are thought to be the primary type of drug/albumin bond. Albumin consists of approximately 56% hydrophobic residues. There is a close correlation between protein binding of a penicillin and its lipid solubility (17). These protein bound compounds have no antimicrobial potential. They can be unmasked by enzyme degradation of albumin or cooking (101). The penicilloyl residues are not detectable by microbial assays but are by immunoassays (if the residue is not bound to albumin) (102). Beta-lactam antibiotics are susceptible to heat inactivation. Pasteurization time and temperatures of milk, however, will not inactivate penicillin residues (67). Using the high temperature short time (HTST) pasteurization temperature of 71C required a holding time of 1705 min to completely inactivate penicillin G.

Beta-lactams are mainly active against gram positive microorganisms. Extremely high concentrations are necessary for activity against gram negative bacteria so they usually are not used for this purpose (85). Beta lactams act by inhibiting bacterial cell wall synthesis as diagramed in Figure 2. Riviere et al. (85) explains that they competitively inhibit the bacterial transpeptidase that cross-links the linear peptidoglycan polymers. This effect is accomplished because the ß-lactam ring is a structural analog of D-alanine/D-alanine that is necessary in the formation of peptide cross-links. The ß-lactams are not effective against gram negative bacteria which have a more complex cell wall with an outer coat of lipoprotein and lipopolysaccharide that the drug must pass through in order to assert an effect.

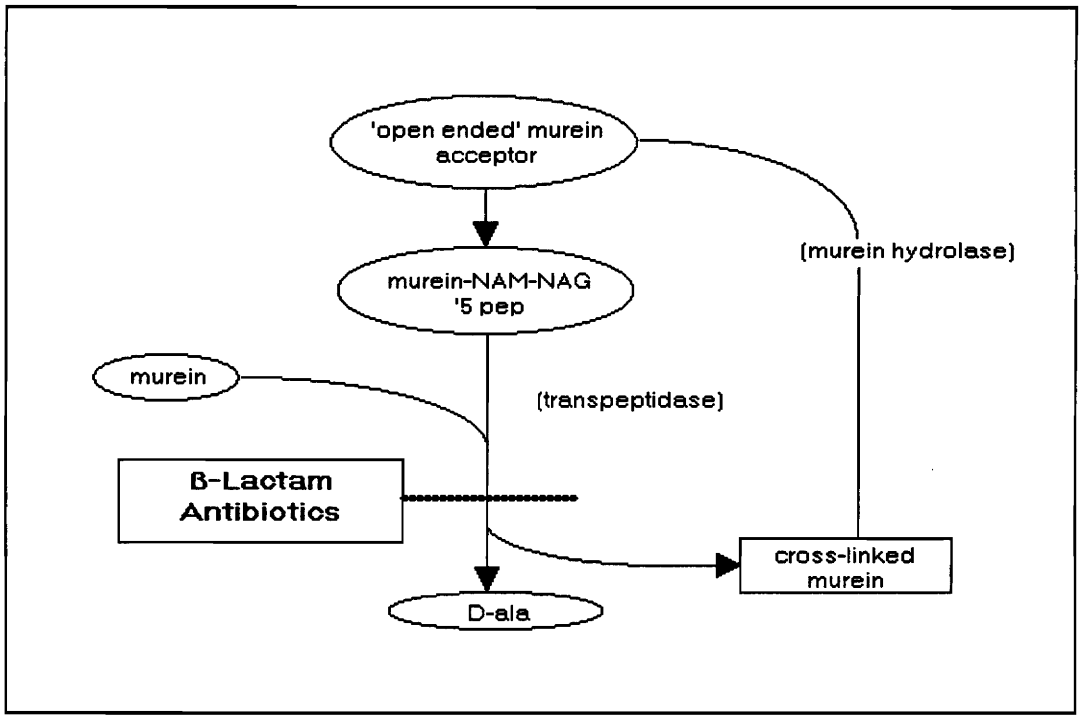

Figure 2. Effect of ß-lactam antibiotics on cell wall synthesis in *E. coli* (81).

Resistance to penicillin has become a common problem. Resistance mechanisms mainly include the production of enzymes that hydrolyze the beta-lactam ring at the C-N bond. This is a plasmid mediated trait but is not passed benignly. It is passed through a bacteriophage during antibiotic therapy (85). Other mechanisms of resistance to penicillin include poor penetration of the antibiotic into the cell, and modification of penicillin binding proteins to prevent or decrease the interaction between drug and protein (34).

Tetracyclines

Tetracyclines are broad spectrum antibiotics. They are natural or semisynthetic and are derived from *Streptomyces*. Chlortetracycline was the first clinically used tetracycline and came into use in 1948. Tetracyclines are amphoteric

zwitterions and will form water-soluble salts with acids and bases. They will form chelate complexes in the presence of divalent and trivalent metals and this can decrease their oral absorption. The presence of dairy products when orally injesting tetracyclines can therefore affect their absorption (85).

Their primary mechanism of action is against prokaryotic protein synthesis. Tetracyclines exhibit their bacteriostatic activity by diffusing through the outer microbial cell membrane, being actively transported across the inner cytoplasmic membrane, and then inhibiting protein synthesis (13,80,85). There are two theories for their bacteriostatic activity identified by Chopra et al. (13). One is that tetracycline molecules bind to ribosomes so codon-anticodon interaction between tRNA and the A site on the ribosome is disrupted. Another is that tetracyclines interrupt the interaction of the elongation factor Tu at the A site, preventing the addition of amino acids to the peptide chain.

This active transport system is one source for plasmid mediated microbial resistance mechanisms and prevents the accumulation of tetracycline in the cell. Reasons for this effect are theorized to include changes in the cytoplasmic membrane and the synthesis of special envelope located proteins. Other mechanisms of resistance cited are increased efflux of tetracycline from the cell, and factors that protect ribosomes. Chopra et al. noted that resistance of organisms to tetracyline is reaching a level that significantly affects its clinical effectiveness (13).

Tetracyclines are not very heat resistant and will also undergo photo decomposition. Katz et al. (46) reported that researchers in Great Britain found that chlortetracycline and oxytetracycline residues were destroyed during cooking. Katz et al. (46) also stated that cooking degraded chlortetracycline to iso-chlortetracycline and oxytetracycline was converted to alpha and beta

apoxytetracyclines. Honikel et al. (39) injected swine with chlortetracycline and oxytetracycline. The researchers found that heating pork in cans at 65C for 60 minutes reduced the chlortetracycline and oxytetracycline to 20% of their original values. They also found that tetracyclines will accumulate in the bones (because of calcium binding) and that the stability of this tetracycline to heat effects is much greater than that of tetracyclines not in bones. Bones needed to be heated to 140C to achieve the same effect as the 65C heat treatment.Freezing was found to have little effect on either drug. A review by Moats (67) cited numerous studies of the stability of tetracyclines to heat in milk. They showed that milk pasteurization processes (71C for 15 seconds) would not inactivate tetracycline residues to a great extent. To completely inactivate chlortetracycline and oxytetracycline at 71C, 280 min and 140 min, respectively, were required. Tetracyclines will form chelate complexes with bivalent ions, like calcium, that are very stable. Suhren and Heeschen (94) noted that tetracyclines bind to plasma proteins as well as calcium. They noted that 55-64% of tetracycline, 45-55% of chlortetracycline, and 20-85% of oxytetracycline in a sample can be bound at one time. They noted that these binding mechanisms will lower the antibacterial activity of the tetracyclines. Treating samples with ammonium oxalate to split complexes and/or protein bonds lowered the limit of detection for Charm II and microbial inhibition tests (94).

Sulfonamides

Sulfonamides have been used clinically for the past fifty years. They were first discovered in the 1930's when the azo dye prontosil was observed to have an effect on microbial growth. It was shown that *in vivo* the protonsil was transformed to sulfanilamide and this substance was the bioactive product (85). All sulfonamides

are derivatives of sulfanilamide. Sulfonamides act by competitively inhibiting dihydropteroate synthetase which condenses pteridine and *p*-aminobenzoic acid (PABA) (81). The sulfonamide competes with PABA. Stopping this pathway prevents the organism from forming folic acid, which is involved in the production of purines, thymine, methionine, constituents of nucleic acids, etc. (81). This is demonstrated in figure 4. Sulfonamides have no effect on preformed folate but this inhibition of folic acid synthesis has a bacteriostatic effect on the organism.

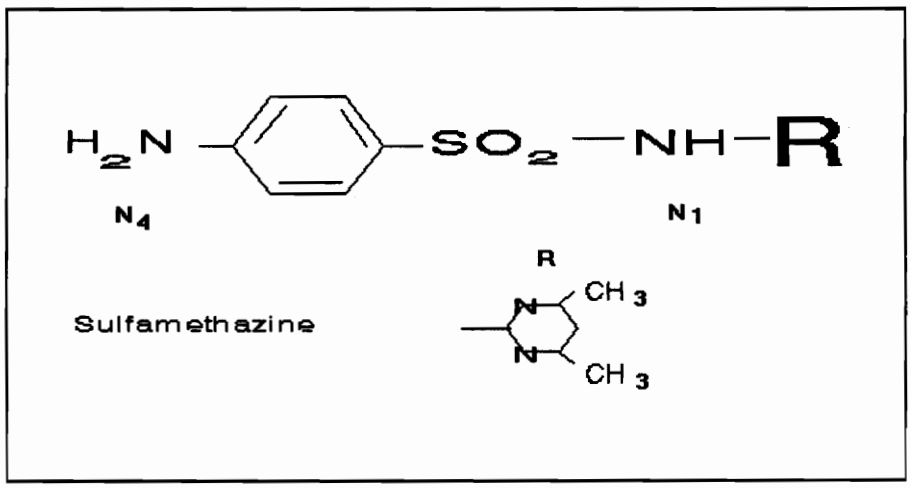


Figure 3. Sulfonamide structure (85).

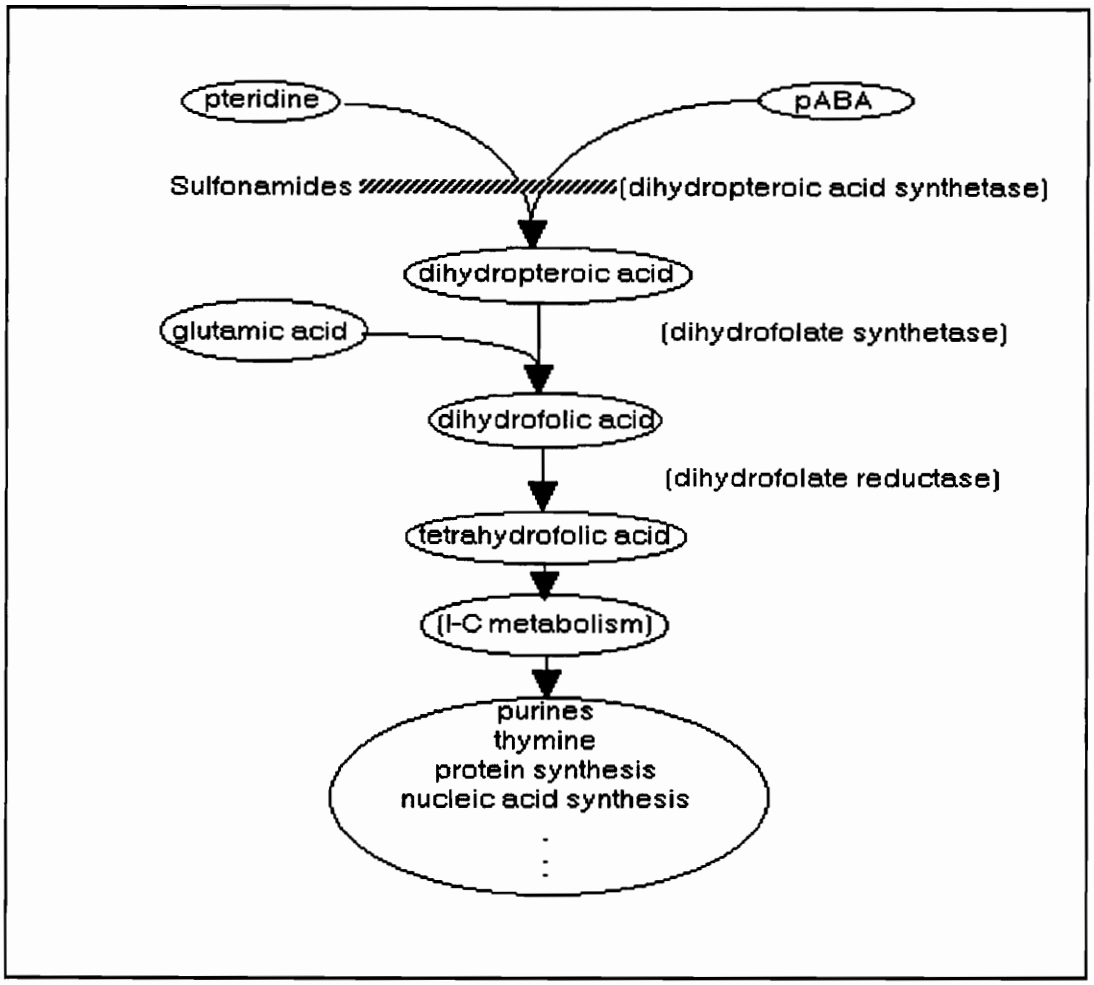

Figure 4. Inhibition of folic acid biosynthesis (81).

Sulfamethazine is an important drug for veterinary use although it is not approved for use in lactating dairy cattle. In spite of this, it is often used extra-labelly as evidenced by many studies (11,15). Sulfamethazine is a sulfapyrimidine and it's chemical name is N'(4,6-dimethyl-2-pyramidinyl)sulfanilamide. Its structure is shown in Figure 3. There has been great public concern recently about sulfamethazine residues in the diet. One concern is that about 3.4% of the population is known to be allergic to sulfonamides (11). Another concern is that sulfamethazine is suspected of causing thyroid cancer in laboratory animals (85). A

final concern is that levels of the drug in food can lead to human pathogenic organisms resistant to sulfamethazine. Mechanisms of resistance to sulfonamides include the increased production of PABA by resistant bacteria, the synthesis of an enzyme that has decreased affinity to sulfonamides, and the synthesis of a second enzyme in the folate synthesis pathway that is resistant to the drugs effect (80).

The main metabolic pathway in sulfonamide metabolism is acetylation of the aromatic amino group in the reticuloendothelial cells of the liver and other tissues to 4-N-acetyl-sulfamethazine. This compound can be converted to more minor compounds such as N-glucose-sulfamethazine and desamino sulfamethazine (106).

Sulfamethazine itself is resistant to various processing effects. Epstein et al. (21) injected swine with sulfamethazine and then processed the meat. They found that the sulfamethazine was not effected by emulsifying ground meat, by curing, or by a combination of curing and heating at 68C or 122C. Small losses of sulfonamides observed prior to curing or heat treatment, were attributed to formation of the 4-N glucopyranosyl derivative. O'Brien et al. (72) injected cattle with sulfamethazine and cooked steaks and roasts from these animals. Sulfamethazine residues were affected either minimally or not at all by either increased temperatures or length of exposure to heat. Sheth et al. (91) found, in studies using sulfathiazole as a representative sulfonamide, that the 4-N aromatic amino group could react with reducing sugars to form different sugar bound products. Sulfathiazole in glucose solution was heated to temperatures ranging from 50C to 80C until the measured amount of sulfathiazole decreased to half of its original content. The time required ranged from 4-10 days. Sulfathiazole could be released from some of these sugar compounds by aqueous dilution or by acidification but Maillard reaction products could not. Microbial inhibition tests,

tests using Bratton-Marshall spray, and possibly microbial receptor assays cannot detect sugar-bound sulfonamides (91). Also, in chromatographic procedures, bound drugs have different mobilities and solubilities. In contrast to the previously described effects on some methods, the researchers found that Amadori-sulfathiazole compound bound better to antibodies in their enzyme-linked immunosorbent assay (ELISA) method than to sulfathiazole itself. Low et al. (57) conducted a similar study of sulfathiazole in honey. They found that sulfathiazole in water held at 80C was stable for over 30 days. When sulfathiazole was put in honey and exposed to similar conditions, 50% was lost in 65 hours. Complete loss of sulfathiazole occurred after 96 hours. Sulfathiazole in honey at room temperature also disappeared but at a slower rate. The sulfathiazole-sugar compound would not diazotize with the Bratton-Marshall reagent because the primary amino group was bound to the sugar.

## METHODS OF ANIMAL DRUG RESIDUE DETECTION

### Microbial Inhibition

Microbial inhibition methods are based on the theory of antibiotic action. The drugs inhibit or slow the growth of microorganisms. Methods tried over the years have been comprehensively reveiwed by numerous authors (6,31,44,45,58,75,88,90). Most of the microbial inhibition assays are developed to detect penicillin, the drug most often used in early dairy cattle mastitis therapy. These assays usually have an organism that is susceptible to low levels of drugs. The organisms that are most often used today are capable of growth at relatively high temperatures so that the speed of the test is increased.

Tests widely used worldwide include disc assay plate methods, cylinder plate methods, and tube assays (44). One official method to detect antibiotics in milk is the *Bacillus stearothermophilus* disc assay method (32,83,97). This method is conducted on agar that is seeded with *Bacillus stearothermophilus* var. *calidolactis* on a petri plate. Paper discs are saturated with sample and placed on the solidified agar. The plates are then incubated at 64C for 2.5 h until zones of inhibition are obtained around a control disc. Agars include Antibiotic Medium No. 4. and PM Indicator Agar (65). Both are accepted by the AOAC although some studies have shown PM Indicator Agar to be more sensitive to penicillin (77,82). The sensitivity range of ß-lactams by this method is 1.6-2.6 ppb. This method will not however determine other classes of drugs at adequate levels of detection. It can only detect tetracyclines and sulfonamides at 400-500 ppb and 100-1,000 ppb, respectively (FDA milk survey results).

Tests that also use agar but by a diffusion ampule method are Delvotest® and Delvotest® P multi (7,47,99). These tests consist of ampules filled with agar and seeded with *Bacillus stearothermophilus* var. *calidolactis*. Sample is placed into the ampule with a nutrient tablet and is incubated. Development of acid by the microorganism causes bromcresol purple in the agar to change to yellow. If antibiotic is present in the sample, no acid will develop and the agar will remain purple. The concentration of penicillin necessary to achieve a positive result was from 3-4.2 ppb. Some studies have found the Delvo tests to be more sensitive than the *B. stearothermophilus* disk assay method, especially when questionable reactions in the Delvo tests are considered positive (31,45,90).

Newer methods based on microbial inhibition but not utilizing traditional agar and zones of inhibition include a reflectance colorimetry method described by

Richardson et al. (84), a bioluminescence method described by Westhoff and Engler (105), and a laser light scattering bioassay described by Wyatt et al. (109). Reflectance colorimetry measures changes in dye pigmentation in a media due to growth of organisms. The bioluminescence method measures the amount of adenosine triphosphate (ATP) produced by organisms in a sample. Both of these methods therefore measure the amount of inhibition in growth of a culture compared to a control and this can be related to the amount of antibiotic in the sample. The laser light scattering method provides measurements that depend upon the average cell size, shape and structure of the culture organisms used. Variations in the cells compared to a control can indicate that antibiotics are present.

Receptor Assays

The microbial receptor assay is based on the fact that animal drug residues will bind to specific sites in sensitive microbial cells such as on the cell wall or intracellular structures. For example, ß-lactams will bind to enzymes on cell walls and tetracyclines will bind to ribosomes. The assay will detect only active drugs, and only classes of drugs. The assay recognizes the functional group of the drug so it cannot differentiate between specific drugs within a class (10). As described in a patent by Charm (9), in the basic embodiment of the test, cell parts containing receptor sites can be immobilized on a support or dried for later reconstititution. The parts are then incubated with sample and labelled animal drug. Animal drug in the sample and tagged drug compete for the binding sites. A separation is made where the bound drug and cell parts are isolated from the unbound. A determination is then made to decide the amount of tagged antibiotic remaining. As the concentration of drug in the sample increases, the amount of tagged drug

that will become bound to the cells decreases. Tags that can be used include $^{14}C$, $^{125}I$, or any other radioactive atom that can be detected by a scintillation counter. Enzymes, enzyme inhibitors, or coenzymes that are detectable when the tagged drug is contacted with a substrate solution are also possible tags.

The microbial receptor assay methods available at the present time are the Charm methods (Charm I, Charm II, Charm Farm), (Penicillin Assays, Inc., Walden, Mass.) The Charm II method is the newest method. It uses radiolabelled drugs in a tablet form and receptor containing cell constituents, also in tablet form. The cell tablet is dissolved in water and either initially incubated with sample alone and then radiolabelled drug in the sequential method, or incubated with sample and radiolabeled drug simultaneously in the competetive method. The sample is then centrifuged and the unbound drug in the liquid is removed. The pelleted material is mixed with scintillation cocktail and then analyzed on a scintillation counter. The counts obtained are compared to a set point. This is determined by taking six measurements of a standard spiked sample provided with the test and adding a certain percentage to the mean of these measurements. Any samples with readings above this number are considered negative and any below are positive.

A number of researchers have evaluated this method. Macauly and Packard (58) found that the Charm I method was the most sensitive of five methods they tested to detect penicillin. Carlsson et al. (8) used the Charm II method to confirm positive microbial inhibition assays and concluded it was a good confirmation method. They noted however that using the fixed percentages for the determination of control points was a danger because the standard deviation varied with the origin of the milk. Suhren and Heeschen (94) noted that tetracyclines form chelates with calcium and bind to plasma proteins. This lowers the antibacterial efficacy of the

tetracycline. Treating milk with ammonium oxalate lowered the limit of detection for Charm II as well as for other tests. They also noted that counts from Charm II varied according to whether the milk was from single cows, a farm or commingled tank milk. The Charm II test for sulfonamides has been noted to detect other PABA analogs like benzoic acid and PABA itself and these can interfere with the test (10,11). Charm et al. (11) noted that feed containing yeast could be a source of PABA.

Immunological Assays

Immune reactions play an important role in the health of man and other animals. They help protect the animal from diseases, or can cause diseases as in allergies. An antibody is a substance that is created in the body in response to an antigen, a compound that is foreign to the body. Antibodies will vary in their specificity for a specific antigen and may cross react with similar antigens from similar sources, i.e., within a drug class (79). Generally, however, antibodies provide a very specific and sensitive method for determining compounds of interest.

There are various methods involving labelling of antigens and antibodies. These include fluorescence, radio and enzyme immunoassays (79). Enzyme immunoassays use enzyme labels and are grouped into homogeneous and heterogeneous categories (19,79). Homogeneous immunoassays require no separation of unreacted reagents. Enzyme-multiplied immunoassay technique (EMIT) is included in this category. Heterogeneous assays have separation and washing steps. An enzyme-linked immunosorbent assay (ELISA) is included in this category. The basic steps in a competetive enzyme immunoassay start with a sample being added to a limited amount of antibody. A labelled antigen is added. Antigen

in the sample and labelled antigen compete with each other for antibody. Unbound antigen is removed and the amount of labelled antigen that bound to antibody is determined. The more antigen there is in the sample, the less the labelled antigen will bind to antibody (79).

Rohner et al. (86) described an ELISA method to detect the benzyl penicilloyl residue of penicillin. This was of use because microbial assays cannot detect this residue.

Dixon-Holland and Katz (20) described an ELISA method they developed to detect sulfamethazine in milk. Milk samples were first acidified and then centrifuged twice. The supernatant was assayed and they were able to estimate sulfamethazine in the range of 1 ppb to 1 ppm.

The Lactek™ methods (Idetek, Inc., San Bruno, CA) are enzyme immunoassays that are developed to detect a variety of drug categories. Plastic test tubes are coated with antibodies to the drug being tested. Sample and enzyme labelled drug are added to the test tube. The antigen in the sample competes with the labelled drug for binding sites on the test tube. The tube is washed to remove unbound antigen and a color developer is added. Any enzyme labelled drug that is remaining will develop a color. The more drug that is present in the sample, the less color will develop. The tubes are then analyzed on a spectophotometer and compared to a standard to determine whether the sample is positive or negative.

The CITE®Probe method (Idexx Corp., Westbrook, ME) is an ELISA method and different tests are available for different drugs. Polyclonal antibodies to the drug being tested are spotted onto a fiber membrane along with a control spot. Sample and enzyme labelled drug are mixed and then applied to the membrane. The drug in the sample competes with the labelled drug for binding sites on the

membrane. Unbound antigen is washed away. An enzyme substrate/chromagen solution is added to the the membrane and color will develop if enzyme labelled drug is present. The more drug that is present in the sample, the less color will develop. The sample spot is compared with the control to determine a positive or negative test.

The Signal® Sulfamethazine Detection Test (SmithKline Beecham Animal Health, Westchester, PA) is an ELISA method to detect sulfamethazine. Microtiter® wells are coated with a capture antibody to sulfamethazine. Sample and enzyme labelled sulfamethazine are added to the wells. Sulfamethazine in the sample competes with the labelled sulfamethazine for binding sites on the wells. The wells are washed to remove unbound sulfamethazine. A substrate is added to react with any remaining enzyme labelled drug to develop a blue color. Samples containing greater than 10 ppb sulfamethazine will prevent the binding of the labelled drug and no color development will occur.

Medina et al. (63) evaluated commercial immunochemical assays for the detection of sulfamethazine in milk. They found that all the tests were capable of detecting sulfamethazine at low ppb levels as the native molecule and possibly the N4-acetyl metabolite. They evaluated Signal, E-Z Quik Card (Environmental Diagnostics, Burlington, NC.), CITE Cup (Idexx, Westbrook, ME.), Lactek, and Agri-screen (Neogen, St. Louis, MO.) for their limits of detection and milk pretreatment effects.

Chromatography

The basic theory behind the chromatographic process is the ability to partition the molecules of a sample between a mobile and a stationary phase (59). It is

desired that sample components of interest will partition to different extents between the mobile and stationary phases so they may be differentiated, identified, and ideally, quantified. The mobile phase can be either a gas, liquid or supercritical fluid, and the stationary phase can be paper, a column of silica, a gel or a variety of other materials. The broad categories of chromatography, named for either their mobile or stationary phase, are thin layer chromatography (TLC), gas chromatography (GC), gel chromatography, and high pressure liquid chromatography (HPLC). In relation to animal drug residue detection, many methods have been tried.

Herbst (35) and Moats (69) reported using TLC methods to identify ß-lactam antibiotics in milk. Moats reported that after some extraction and purification steps, an aliquot was spotted on silica gel plates that were developed with chloroform-acetone-glacial acetic acid. The ß-lactams were visualized after development by spraying with 1N HCL, then starch, then exposing to iodine vapor to form intense blue/black spots. The limit of detection for the penicillin G standard was about 4 ppb. Herbst (35) related that after an agar well diffusion technique as a preliminary screening, positive samples were subjected to TLC on silica gel plates. The completed TLC plates were then subjected to bioautography by flooding the TLC plate with antibiotic Medium 36, that had been seeded with *B. stearothermophilus,* and were incubated overnight. The trays were sprayed with 2-(*p*-iodophenyl)-3-*p*-nitrophenyl-5-phenyl tetrazolium chloride and incubated until the zones of inhibition were clear. Kondo (48) related a method of TLC-bioautography to differentiate antibiotics. Antibiotic solutions of suitable concentration were spotted on TLC plates and developed in seven different, graded concentrations of ammonium chloride. Dried, developed plates were placed on agar seeded with *B.*

*subtilis* or *M. luteus* and incubated overnight at 37C. The minimum amount of penicillin G and tetracycline detected was 2 ppb and 25ppb $\mu$g respectively. Roybal et al. (87) and Unruh et al. (98) described methods of solid-phase extraction of sulfamethazine, followed by TLC. Unruh reported that diluted homogenized milk samples were passed through a C18 column. The sulfamethazine was eluted with methanol and purified and concentrated by being passed through two more columns. The concentrate was then applied to silica gel TLC plates. Fluorescence was induced with fluorescamine and the drug was quantitated with a scanning densitometer. Good recoveries were reported in the analysis range of .51-15 ppb. Roybal (87) reported passing diluted milk through a solid phase extraction column and eluting the sulfamethazine with acetonitrile. The sulfamethazine was concentrated then spotted and run on a TLC plate. The sulfamethazine was diazotized with sodium nitrite and visualized with 0.1% N-1-naphthylenhylene diamine dihidrochloride in methanol. Clark et al. (14) related a method to detect eight sulfonamides in milk. Defatted milk was introduced into an Extrelut column. Sulfonamides were eluted with methylene chloride. The sample was evaporated, dissolved in chloroform, spotted, and run on a TLC plate. The sulfonamides were diazotized and visualized by the method previously described. Sensitivity was reported to be at or below 10 ppb. Some sulfonamides had similar Rf values and two different solvents were needed to get complete separation.

Lott et al. (56) described a method of high voltage electrophoresis bioautography. Milk was either analyzed by high voltage electrophoresis in agar gel directly or, if low concentrations of antibiotics were being measured, was extracted with acetonitrile and water. An overlayer of the electrophoresis gel was made with antibiotic medium No. 1 seeded with *M. luteus* or *B. cereus*. After an incubation, the

presence of antibiotics was visualized by a zone of inhibition being present. Migration distances and inhibition zone appearance were compared to standards, and preliminary microbial screening results were assessed so that identification of the antibiotic was made.

Meetschen and Petz (64) described a method to determine ß-lactam residues by capillary gas chromatography. Residues were extracted and cleaned, then methylated with diazomethane so they were detectable by gas chromatography on a methyl silicone fused column. The residues were detected by a nitrogen-selective thermoionic detector. The quantitation level was reported to be 3 ppb and limit of detection in the sub-ppb range.

The most cited method of chromatographic determination of animal drug residues is HPLC. In column liquid chromatography, the solid phase is held in a column and the mobile phase is allowed to flow or is pumped over it (59). In HPLC, the liquid is pumped, under pressure, through the column. Many researchers describe methods for the determination of tetracyclines, ß-lactams and sulfonamides by HPLC (1,2,3,4,5,23,49,52,53,54,55,66,68,70,76,92,93,95,96,104). Macrae (59) noted that in testing for small levels of an analyte, it is not the resolving power of the HPLC method that is inadequate, but the elaborate extractions that are often required to isolate the analyte in a pure enough state to achieve high recovery. Barker et al. (5) pointed out that the classical approach to preparing samples for analysis involves the following: mincing and/or mechanical homogenization of the tissue in an aqueous solvent; addition of acids, bases or salts to precipitate protein and remove cellular debris; centrifugation; transfer of super natant and adjustment of pH; counter-current extraction of the sample, often leading to formation of emulsions; and back-extraction to assist in purification of the

sample. Disadvantages to this system are stated as being time consuming, resulting in less than ideal recoveries, loss of accuracy and/or precision, and possibly disallowing multi-residue isolation within a drug class from one sample (55). Barker et al. (5) first described a method of using a lipophilic solid phase packing material (an octadecylsilyl derivatized silica known as C18) to disperse tissues onto a solid support. They described this approach, matrix solid phase dispersion (MSPD), as follows. Tissue is blended with C18 coated silica beads by using a mortar and pestle. The disrupting of cell membranes by solubilization of the lipid and cholesterol components into the polymer matrix, with more polar constituents directed outward is thought to occur. This essentially unfolds the structural components of the tissue. Scanning and transmission electron micrographs of tissue coated C18 packing show that the process totally disrupts the organelle structure. Since the sample is evenly distributed on a solid support of great surface area, (1000 $m^2$/2g of C18) the entire sample is exposed to extraction agents (55). Long et al. (52) noted that components in milk (lipids, carbohydrates, proteins, salts, etc.) contribute a unique chemical characteristic to the column bed. Simply passing milk through a C18 cartridge does not result in consistent recoveries of sulfonamides. Milk does not sufficiently coat particles unless it is mechanically blended into the material. Barker et al. (5) described a method using MSPD followed by HPLC for detection of ß-lactam antibiotics in tissues. Long et al. (52) described a method using MSPD followed by HPLC using UV detection for detection of eight sulfonamides in milk. They noted their limit of detection as being between 31.25 and 62.5 ppb. Long et al. (54) described a method using MSPD followed by HPLC with UV detection to determine oxytetracycline, tetracycline, and chlortetracycline in milk. Both ethylinediaminetetraacetate (EDTA) and oxalic acid were required when preparing

the C18 column to get good recoveries of tetracyclines because tetracyclines tend to form complexes with inorganic ions. EDTA chelates the inorganic ions and apparently releases tetracyclines from the complex. The oxalic acid alters the pH and changes extraction characteristics.

# CHAPTER 3

# MATERIALS AND METHODS

## MILK COLLECTION AND PROCESSING

Raw milk was obtained from three first-calf holstein cows from the Virginia Tech Dairy farm. They had never been treated with animal drugs. The milk was split into two lots. One that was drug free (not spiked) and one with concentrations of either sulfamethazine, chlortetracycline or penicillin added to it (spiked). After adding drug solutions to achieve concentrations of 10 ppb, 30 ppb, and 10 ppb, respectively (Appendices A, B and C) these lots were split into sublots to receive different treatments of heat treatment, homogenization, both heat treatment and homogenization, or no treatment (Figure 5). Samples that were heat treated were heated in batch in bottles at 63C for thirty minutes. Homogenized samples were processed at 49C on an APV two stage homogenizer with the first stage at 500 psi and the second state at 1,000 psi (APV Gaulin Model 15 MR 15, Everett, MA USA). Three replicates of each animal drug residue were made and five subsamples taken for each processing combination for each test method (excluding HPLC).

For drug residue detection by HPLC analysis, one sample was taken of each spiked treatment, for all drugs, for each replicate. The samples were analyzed by Environmental Systems Services, Culpeper, Virginia.

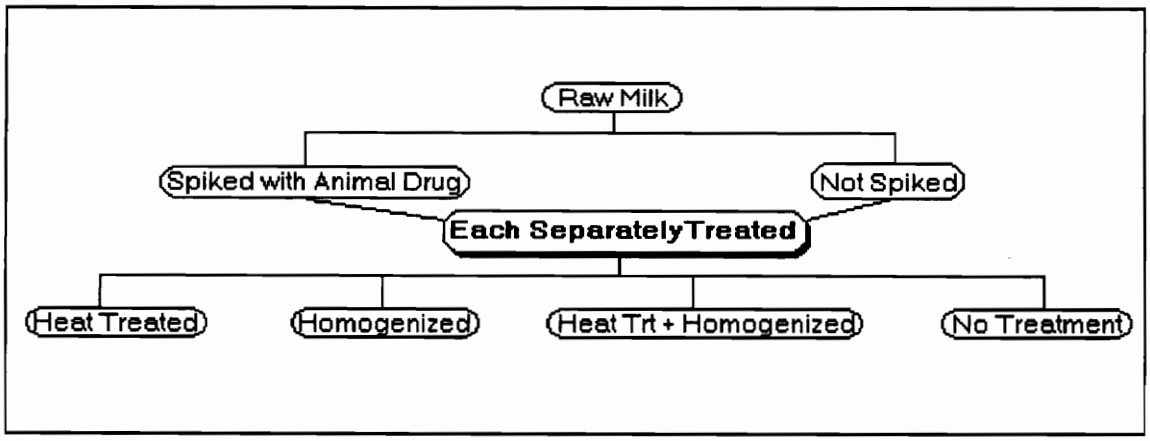

Figure 5. Method Outline.

### DRUG RESIDUE DETECTION

Samples containing sulfamethazine and control samples were tested by CITE, Signal, Charm II, Lactek, and HPLC methods. The procedures for these tests are described in appendices H, I, K, D, respectively,and the method of Long et al. (52). Samples containing chlortetracycline and control samples were tested by CITE, Charm II, and HPLC. The procedures for these tests are described in appendices G, L, respectively, and Long et al. (54). Samples containing procaine penicillin G and control samples were tested by CITE, Charm II, Lactek, *Bacillus stearothermophilus* disk assay, and HPLC. The procedures for these tests are described in appendices F, J, E, M, respectively, and Barker et al. (5).

### STATISTICAL EVALUATION

Most of the screening methods give a qualitative "yes" or "no" response for the drug being tested. Statistical analysis was conducted on all tests by the SAS CATMOD procedure for analyzing this categorical data (SAS Institute, Inc., Cary,

NC). The treatments of the drug residues tested were compared within spiked and non-spiked samples for significant effects at a $p < .05$ and blocked on the replicate. The Charm II method gives "counts per minute" (cpm) readings on a scintillation counter and quantification of an identified drug using a standard curve is possible. The Charm II method identifies drug classes, not a specific drug. For these reasons, the Charm II data was also tested using the ANOVA procedure on the cpm raw data (SAS Institute, Inc., Cary, NC). Treatment effects were compared for each drug tested and were compared within the spiked and non-spiked samples for significant effects at a $p < .05$. Blocking was conducted on the replications. Significant treatment effects were compared using Duncan's Multiple Range Test to determine differences.

# CHAPTER 4

# RESULTS AND DISCUSSION

The ability to monitor antibiotics and animal drugs on the dairy farm and at the processing plant is essential to obtain a drug free milk supply. Tests on the farm and most tests conducted in the dairy plant are conducted on raw milk. In order to survey the milk supply for drug residues, however, some state agencies are using these test methods on finished milk products. All these methods were developed for use, and found to be effective, on raw milk. The ability of these tests to detect drug residues effectively in processed milk needed to be determined.

## PENICILLIN RESULTS

Table 1. Summary of penicillin detection.

| METHODS | NO TRT | | HT TRT | | HOMO | | HT/HOMO | |
|---|---|---|---|---|---|---|---|---|
| | S | NS | S | NS | S | NS | S | NS |
| Charm II | 15 | 0 | 12 | 0 | 10 | 0 | 15 | 0 |
| CITE Probe | 8 | 0 | 13 | 0 | 12 | 0 | 7 | 0 |
| Disk Assay | 15 | 0 | 14 | 0 | 13 | 0 | 15 | 0 |
| LacTek | 15 | 2 | 15 | 0 | 15 | 5 | 15 | 1 |

# out of 15 samples positive for drugs.
S = spiked sample
NS = not spiked sample

No significant treatment effects were found using the CATMOD procedure on either spiked or nonspiked milk for all test methods, excluding HPLC. The summary of the data responses can be seen in Table 1. The control point used for the microbial receptor assay data was 513. Using the ANOVA procedure on the microbial receptor assay data, significant results were found for both spiked and non-spiked milk (Tables 2 and 3). For spiked milk, treatment, blocking on the replicate and treatment/replicate interactions were all significant. For non-spiked milk, only the treatment was significant. The coefficients of variation were 8.1 and 7.0 and the overall R-squares and F values were .93 and .24 and 53.56 and 1.39 for the spiked and non-spiked samples, respectively. Using Duncan's Multiple Range Test as a means separation method, in spiked milk, it can be seen that both homogenized, and heat treated samples varied significantly from raw and homogenized/heat treated samples (Table 4). In control milk tested for beta-

lactams, it can be seen from the Duncan groupings that there were no clean separations in the mean groupings (Table 4). This is due to the lack of overall significance (F value of 1.39) found by the ANOVA procedure. The HPLC data was not analyzed statistically due to enormous variances in the results without any discernable pattern.

**Table 2. ANOVA Table for penicillin spiked sample tested by the microbial receptor assay.**

| Source | DF | ANOVA Sum of Squares | F Value | PR > F |
|---|---|---|---|---|
| Trt | 3 | 57440.9 | 20.49 | .0001 |
| Replication | 2 | 391119.63 | 209.3 | .0001 |
| Trt*Rep | 6 | 101922.4 | 18.18 | .0001 |

**Table 3. ANOVA table for non-spiked milk tested for beta-lactams by the microbial receptor assay.**

| Source | DF | ANOVA Sum of Squares | F Value | PR > F |
|---|---|---|---|---|
| Trt | 3 | 40051.6 | 2.83 | .048 |
| Replication | 2 | 9787.6 | 1.04 | .3618 |
| Trt*Rep | 6 | 22084.5 | 0.78 | .5889 |

**Table 4. Duncan's Multiple Range Test of penicillin samples tested by the microbial receptor assay.**

| Treatment | Spiked | Non-spiked |
|---|---|---|
| Raw | 355.33$^{c}$ | 1013.13$^{a}$ |
| Homo | 417.67$^{a}$ | 985.33$^{ab}$ |
| Heat Trt | 392.2$^{b}$ | 957.13$^{b}$ |
| Homo/Heat Trt | 338.53$^{c}$ | 946.87$^{b}$ |

n = 15
a,b,c means with different letters indicate means are significantly different at $p < .05$.

## CHLORTETRACYCLINE RESULTS

**Table 5. Summary of chlortetracycline detection.**

| METHODS | NO TRT | | HT TRT | | HOMO | | HT/HOMO | |
|---|---|---|---|---|---|---|---|---|
| | S | NS | S | NS | S | NS | S | NS |
| Charm II [a] | 15 | 0 | 14 | 1 | 15 | 0 | 15 | 0 |
| CITE Probe[a] | 12 | 2 | 14 | 1 | 13 | 0 | 15 | 0 |

# out of 15 samples positive for drugs.
S = spiked sample
NS = not spiked sample

No significant treatment effects were found using the CATMOD procedure on either spiked or nonspiked milk. The summary of the data can be viewed in Table 5. The control point used in the microbial receptor assay data was 1271. Using the ANOVA procedure on the microbial receptor assay data, no significant treatment effects were found for either spiked or non-spiked milk. Blocking on the

replication was effective for both spiked and non-spiked samples and a significant treatment/replicate interaction was found for the non-spiked samples (Tables 6 and 7). The coefficients of variation were 8.5 and 8.8 and the overall R-squares and F values were .67 and .76, and 9.06 and 14.75 for the spiked and non-spiked samples, respectively. The HPLC results were not analyzed statistically due to the frozen samples spoiling before they were tested and no chlortetracycline being detected.

**Table 6. ANOVA Table for chlortetracycline spiked sample tested by the microbial receptor assay.**

| Source | DF | ANOVA Sum of Squares | F Value | PR > F |
|---|---|---|---|---|
| Trt | 3 | 56727.5 | 2.51 | .069 |
| Replication | 2 | 614236.2 | 40.82 | .0001 |
| Trt*Rep | 6 | 79260.4 | 1.76 | .1286 |

**Table 7. ANOVA table for non-spiked milk tested for chlortetracycline by the microbial receptor assay.**

| Source | DF | ANOVA Sum of Squares | F Value | PR > F |
|---|---|---|---|---|
| Trt | 2 | 82734.4 | 1.14 | .3315 |
| Replication | 2 | 3573002.7 | 49.18 | .0001 |
| Trt*Rep | 4 | 631031.8 | 4.34 | .0057 |

## SULFAMETHAZINE RESULTS

Table 8. Summary of sulfamethazine detection.

| METHODS | RAW | | HT TRT | | HOMO | | HT/HOMO | |
|---|---|---|---|---|---|---|---|---|
| | S | NS | S | NS | S | NS | S | NS |
| Charm II | 11 | 0 | 7 | 0 | 13 | 0 | 7 | 0 |
| CITE | 15 | 0 | 15 | 0 | 14 | 1 | 13 | 0 |
| Signal | 15 | 0 | 15 | 0 | 15 | 0 | 15 | 0 |
| LacTek | 15 | 1 | 13 | 0 | 12 | 2 | 15 | 1 |

# out of 15 samples positive for drugs.
S = spiked sample
NS = not spiked sample

The summary of the raw data results can be seen in Table 8. The results found by using the CATMOD categorical data analysis showed that there were no significant treatment effects for any of the test methods (excluding HPLC). The control point used for the microbial receptor assay data was 1113.6. Using the ANOVA procedure on the microbial receptor assay data, significant results were found for both spiked and non-spiked milk (Tables 9 & 10). Treatments, blocking on the replicate and a treatment/replicate interaction were all significant. The coefficient of variation was 7.5 for both spiked and non-spiked and the overall R-square and F value was .69 and .94, and 9.57 and 69.73 for the spiked and non-spiked, respectively. Using Duncan's Multiple Range Test as a means separation method, in spiked milk, both heat treated, and homogenized and heat treated milk varied significantly from the other treatments (Table 11). That is, the heat treated

milks varied significantly from the other treatments. In control milk tested for sulfonamides both heat treated and homogenized treatments varied significantly from homogenized/heat treated and raw samples (Table 11). HPLC data was not analyzed due to all but one result not falling within the 60-110% recovery desired in analytical methods in the ppb range.

**Table 9. ANOVA Table for sulfamethazine spiked sample tested by the microbial receptor assay.**

| Source | DF | ANOVA Sum of Squares | F Value | PR > F |
|---|---|---|---|---|
| Trt | 3 | 339826.7 | 17.98 | .0001 |
| Replicate | 2 | 198056.6 | 15.72 | .0001 |
| Trt*Rep | 6 | 125681.4 | 3.32 | .0081 |

**Table 10. ANOVA table for non-spiked milk tested for sulfonamides by the microbial receptor assay.**

| Source | DF | ANOVA Sum of Squares | F Value | PR > F |
|---|---|---|---|---|
| Trt | 3 | 21349185.9 | 206.35 | .0001 |
| Replicate | 2 | 1216915.6 | 17.64 | .0001 |
| Trt*Rep | 6 | 3888512.8 | 18.79 | .0001 |

**Table 11. Duncan's Multiple Range Test of sulfamethazine samples tested by the microbial receptor assay.**

| Treatment | Spiked | Non-spiked |
|---|---|---|
| Raw | 986.2[b] | 2669.53[b] |
| Homo | 966.97[b] | 1492.13[c] |
| Heat Trt | 1136.53[a] | 3088.27[a] |
| Homo/Heat Trt | 1114.47[a] | 2683.8[b] |

n = 15
a,b,c means with different letters indicate means are significantly different at $p < .05$.

## DISCUSSION

For the microbial receptor assay, some significant treatment effects were found. In the case of sulfamethazine, heat treatment of the milk caused higher readings than when the same milk was tested raw. This could lead to an increased risk of calling a sample that is positive, when tested raw, negative. These processing effects were evident whether or not there was drug present in the milk. Heat treating the milk therefore affected some component in the milk and allowed more radio-labelled sulfonamide to bind and remain to be counted at the end of testing. The labelled drug could possibly be binding to microorganisms present in the milk sample, or denatured serum proteins. The sulfonamide/reducing sugar interactions that were described by Sheth et al. (91) and Low et al. (57) do not appear to be occurring to a great enough extent to affect the test. These studies, indicating heat treatment effects on detection of sulfonamides, used higher temperatures and longer durations than were used in this study. There could be a slight sulfonamide/sugar interaction as evidenced by the F value for the spiked milk treatment effect being much lower than for the unspiked. In the case of milk spiked

with penicillin, homogenization and heat treatment both caused an increase in the mean cpm counts over raw and homogenized/heat treated milk. Since these same effects were not observed to the same extent on non-spiked milk, these treatments seem to be affecting the added drug and not the milk itself. Penicillin has been shown to bind extensively to blood serum albumin proteins. Fifty-six percent of albumin consists of hydrophobic residues (17). Penicillins contain various levels of lipid solubility. The strength of hydrophobic bonds is increased with heat so possibly more penicillin is bound to albumin after heat treatment and not detected. The penicillin may also possibly be binding to denatured serum proteins. Homogenization greatly increases the surface area of the fat globules. Milk fat globule membranes are unable to surrond all the fat so a large amount of casein becomes adsorbed to the surface of the globule. The penicillin may then possibly adsorb to this casein that is altered in structure. This could explain why homogenization appeared to decrease the amount of spiked penicillin detected. Neither of these effects explain, however, why the combined homogenization/heat treatment sample exhibited a significantly lower value than the separate heat treated and homogenized samples. Chlortetracyline exhibited no significant treatment differences by the microbial receptor assay method. Tetracyclines in serum associate with lipoproteins (17). Homogenization will increase the amount of lipoproteins exposed on the surface of fat globules. The increased availability of lipoproteins to tetracycline binding didn't appear to effect the test. Tetracyclines will also bind to calcium but the calcium in the milk didn't appreciably affect the amount of tetracycline detected.

Carlsson et al. (8) noted that the choice of the zero milk is critical because it is used as an estimate to which the control point percentages are applied. In

sulfonamide detection, since there seems to be a heat treatment effect on the milk, it may be advantageous to use heat treated milk for the control point determination. Although these treatment effects were found significant with the analysis of variance procedure, when the control point was determined to decide when a sample is considered positive or negative (as outlined in the Charm II methodology), the treatment effects washed out. There were no significant treatment differences for any of the drugs tested when the samples were called positive or negative and then evaluated by categorical data analysis. The heat treatment employed in this study and homogenization therefore did not affect whether a test was correctly called positive or negative.

The results for the rest of the methods, excluding HPLC, using categorical data analysis, showed no significant treatment effects. This does not suggest an absence of processing effects on these methods. But, based on how the tests are run and interpreted, there were no significant effects found due to the heat treatment, homogenization or a combination of both.

No conclusions could be drawn about treatment effects on the HPLC methods because the method did not seem to work properly. This was also observed in a previous study conducted at Virginia Tech.

All test methods used in this study, with the exception of HPLC, worked properly when milk was heat treated, homogenized, or received a combination of both treatments. Treatment effects with penicillin and sulfamethazine spiked milk show the need to test each method on a specific drug by drug basis if a different, more severely treated milk product is going to be tested.

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# APPENDIX A

## CALCULATION METHOD FOR MAKING A 10 PPB SULFAMETHAZINE MILK SOLUTION.

10 mg of powdered, 100% pure sulfamethazine (Sigma Chemical Corp.) was suspended and brought to 10 ml volumetrically in 200 proof ethanol to produce solution A.

1 ml of solution A was brought to 1 L with animal drug free milk to produce solution B.

50 ml of solution B was then brought to 5L volumetrically with animal drug free milk to produce a 10ppb sulfamethazine in milk solution.

This solution was made fresh for each replicate.

# APPENDIX B

## CALCULATION METHOD FOR MAKING A 30 PPB CHLORTETRACYCLINE MILK SOLUTION.

10 mg of powdered 80% pure chlortetracycline was brought to 10 ml volumetrically with dairy dilution buffer (standard methods) to produce solution A.

1.25 ml of solution A was brought to 1L volumetrically in animal drug free milk to produce solution B.

150 ml of solution B was brought to 5L with animal drug free milk to produce a 30ppb chlortetracyline milk solution.

This solution was made fresh for each replicate.

# APPENDIX C

## CALCULATION METHOD FOR MAKING A 10 PPB PENICILLIN MILK SOLUTION .

10mg procaine penicillin G (Sigma Chemical) was brought to 10 ml volumetrically with dairy dilution buffer (standard methods) to make solution A.

1.7 ml of solution A was brought to 1L volumetrically with animal drug residue free milk to produce solution B.

50 ml of solution B was brought to 5L volumetrically with animal drug residue free milk to produce the 10ppb milk solution.

This solution was made fresh for each replicate.

# APPENDIX D

## LACTEK™ METHOD FOR SULFAMETHAZINE DETERMINATION .

Materials and procedures provided in Sulfamethazine Milk Screening Kit (Catalog No. 8040) by Idetek, Inc., San Bruno, CA:

| | |
|---|---|
| Reaction Tubes | -Coated with antibody to sulfamethazine |
| Standard | -Sulfamethazine in buffer |
| Solution 1 (tracer) | -Horseradish peroxidase-sulfamethazine conjugate with stabilizer, lyophilized |
| Diluent for tracer | -Phosphate buffered saline with preservative |
| Solution 2 (color developer) | -2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)(ABTS) and hydrogen peroxide in citrate buffer |
| Solution 3 (stop solution) | -Dilute sodium dodecyl sulfate |
| Wash concentrate | -Saline and surfactant to be diluted with deionized water |

Additional Equipment:

Spectrophotometer

Tube washing apparatus - Jet washer or squirt bottle

pipettes or reagent dispensers

timer

Test Procedure:

1. 250 $\mu$l of Standard is added to an antibody coated reaction tube.
2. 250 $\mu$l of milk is added to other antibody coated reaction tubes.
3. 250 $\mu$l of reconstituted Solution 1 is added to all tubes. Tubes are incubated for 3 to 4 minutes at room temperature while shaking.
4. Each tube is washed by inverting over the nozzle of the Jet Washer for three seconds. All of the wash solution is then shaken out until no moisture remains in the tubes.
5. 500 $\mu$l of Solution 2 is added to each tube. The tubes are allowed to incubate at room temperature for 3-4 minutes while shaking.
6. 500 $\mu$l of Solution 3 (Stop Solution) is added to each tube. The tubes are read with a spectrophotometer set at 405 nm. The optical density of each sample tube is compared with that of the tube containing the Standard.

If the optical density of the sample is greater than or equal to the optical density of the Standard, then the sample is considered negative for sulfamethazine. If the optical density of the sample is less than the optical density of the Standard, the sample is considered positive for sulfamethazine.

# APPENDIX E

## LACTEK™ METHOD FOR BETA-LACTAM DETERMINATION .

Materials and procedures provided in Beta-lactam Milk Screening Kit (Catalog No. 8041) by Idetek, Inc., San Bruno, CA.:

| | |
|---|---|
| Reaction Tubes | -Coated with antibody to beta-lactam |
| Standard | -Beta-lactam in buffer, lyophilized |
| Solution 1 (tracer) | -Horseradish peroxidase-beta-lactam conjugate with preservative, lyophilized |
| Diluent for tracer | -Phosphate buffered saline with preservative |
| Solution 2 (color developer) | -2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)(ABTS) and hydrogen peroxide in citrate buffer |
| Solution 3 (stop solution) | -Dilute sodium dodecyl sulfate |
| Wash concentrate | -Saline and surfactant to be diluted with deionized water |

Additional Equipment:

Spectrophotometer

Tube washing apparatus - Jet washer or squirt bottle

pipettes or reagent dispensers

timer

Test Procedure:

1. 250 $\mu$l of Standard is added to an antibody coated reaction tube.
2. 250 $\mu$l of milk is added to other antibody coated reaction tubes.
3. 250 $\mu$l of reconstituted Solution 1 is added to all tubes. Tubes are incubated for 3 to 4 minutes at room temperature while shaking.
4. Each tube is washed by inverting over the nozzle of the Jet Washer for three seconds. All of the wash solution is then shaken out until no moisture remains in the tubes.
5. 500 $\mu$l of Solution 2 is added to each tube. The tubes are allowed to incubate at room temperature for 3-4 minutes while shaking.
6. 500 $\mu$l of Solution 3 (Stop Solution) is added to each tube. The tubes are read with a spectrophotometer set at 405 nm. The optical density of each sample tube is compared with that of the tube containing the Standard.

If the optical density of the sample is greater than or equal to the optical density of the Standard, then the sample is considered negative for beta-lactams. If the optical density of the sample is less than the optical density of the Standard, the sample is considered positive for beta-lactams.

# APPENDIX F

## CITE PROBE METHOD FOR BETA-LACTAM DETERMINATION .

CITE® Probe® kits, Idexx Corp., Westbrook , ME, for the detection of beta-lactam antibiotics contain all reagents pre-measured in four sample wells and procedures for the test. An optical reader and recorder is optional.

Method:

1. Nine drops of sample are added to sample well number 1 with a provided dropper. The tray is shaken and allowed to incubate at room temperature for 10 minutes.
2. The sample is shaken again and the provided sample probe is inserted into well 1 and incubated at room temperature for 1 min.
3. The probe is removed from the well and the blue button on the sample probe is pushed in firmly. The pre-filter remains in well 1 and the probe is inserted into well 2. It is incubated for 1 min at room temperature.
4. The probe is transferred to well 3 and incubates for 30 sec at room temperature.
5. The probe is transferred to well 4 and incubates for 10 sec at room temperature.
6. The probe is removed and allowed to sit at room temperature for approximately one minutes so that color can develop on the two spots on the end of the probe.
7. After blotting, the probe is inserted into a device that reads the difference in color between the control spot and the sample spot.

A control spot that is equal to or lighter than a sample spot is considered negative to beta-lactams. A control spot that is darker than a sample spot is considered positive. This test detects to 5 ppb penicillin G.

# APPENDIX G

## CITE PROBE METHOD FOR TETRACYCLINE DETERMINATION .

CITE® Probe® kits, Idexx Corp., Westbrook, ME, for the detection of tetracyclines contain all reagents pre-measured in four sample wells and procedures for the test. An optical reader and recorder is optional.

Test Procedure:

1. Four drops of sample are added to sample well number 1 with a provided dropper. The tray is shaken and allowed to incubate at room temperature for 1 minute.
2. The provided sample probe is inserted into well 1 and incubated at room temperature for 1 min.
3. After removing the probe, the blue button on the sample probe is pushed in firmly. The pre-filter remains in well 1 and the probe is inserted into well 2. It is incubated for 1 min at room temperature.
4. The probe is transferred to well 3 and incubated for 30 sec at room temperature.
5. The probe is transferred to well 4 and incubated for 15 sec at room temperature.
6. The probe is removed and allowed to sit at room temperature for approximately 1 min so that color can develop on the two spots on the end of the probe.
7. After blotting, the probe is inserted into a device that reads the difference in color between the control spot and the sample spot.

A control spot that is equal to or lighter than a sample spot is considered negative to tetracyclines. A control spot that is darker than a sample spot is considered positive. This test detects chlortetracycline to 40 ppb.

# APPENDIX H

## CITE SULFA TRIO™ METHOD OF SULFAMETHAZINE DETERMINATION .

Materials and procedures provided in the CITE® Sulfa Trio™ Test kit, IDEXX Corp., Westbrook, ME:

| | |
|---|---|
| CITE Devices with prefilters | |
| Wash solution | -Preserved with Gentamicin |
| Solution 1 Sulfa Drug preservative | -Alkaline Phosphatase Conjugate and |
| Solution 2 | -Substrate Solution preserved with sodium azide |
| Solution 3 | -Stop Solution |
| pipets | |
| Sample cups | |
| Filter seating tool | |

Test procedure:

1. The CITE device is wet with 10-15 drops of Wash Solution. The prefilter is pressed with the seating tool.

2. Six drops of sample are added to the sample cup. Six drops of Solution 1 are added to the sample cup. The cup is swirled to mix and poured into the CITE device. It is allowed to incubate at room temperature for 3 min.

3. The prefilter is removed and the CITE device is washed by allowing 10-15 drops of wash to be absorbed, then filling the CITE device with wash and allowing this to be absorbed.

4. Three drops of Solution 2 are added to the CITE device and it is incubated at room temperature for 2 min.

5. Ten to 15 drops of Solution 3 is added to stop the reaction.

Four spots are present on the CITE device. One is a control and the other three are spots for sulfadimethoxine, sulfathiazole, and sulfamethazine. When a sample spot is darker than the control spot, the sample is negative for that sulfa drug. When a sample spot is equal to or greater than the control spot, the sample is a presumptive positive for that sulfa residue. The test detects 5 ppb or greater of the sulfa residues.

# APPENDIX I

## SIGNAL METHOD FOR SULFAMETHAZINE DETECTION .

Materials and procedures provided with Signal® Sulfamethazine Detection Kit, SmithKline Beecham Animal Health, Westchester, PA:

Antibody coated Microtiter® well strips

Sulfamethazine Enzyme Conjugate

Removawell® strip holder to hold eight 12 well strips

Substrate A

Substrate B

Dilution Buffer

Wash Solution

Test procedure:

1. 20 $\mu$l of sample is added to a test well.
2. 100 $\mu$l of sulfamethazine enzyme conjugate is added to each sample well and it is mixed. It is incubated at room temperature for 20 min.
3. Sample wells are dumped and washed three times with wash solution. They are tamped dry so no moisture remains.
4. 150 $\mu$l of substrate is added to each well. This is made by mixing equal amounts of substrate A and B. It is incubated at room temperature for 5 min.

Results are read by noting the presence or absence of blue color in the sample. If a blue color change occurs, the sample contains less than 10 ppb sulfamethazine and

is considered negative. If no color change occurs, the sample contains more than 10 ppb sulfamethazine and is considered positive.

# APPENDIX J

## CHARM II METHOD FOR BETA -LACTAM DETERMINATION .

Materials and procedures provided with Charm II beta-lactam kit by Penicillin Assays Inc., Malden MA.:

| | |
|---|---|
| Reagent A | -($^{14}$C)penicillin |
| Reagent B | -Binding Reagent |
| Positive Control Standard | -5 ppb penicillin G standard |

Other materials:

| | |
|---|---|
| Scintillation counter with $^{14}$C channel | -Charm II reader |
| Scintillation cocktail | -Packard Opti-flour |
| pipettes | |
| distilled water | |
| test tubes | |
| centrifuge | |
| heating block | |
| vortex mixer | |
| cotton swabs | |

Test Procedure:

1. Add a Reagent B tablet added to a test tube.
2. Three hundred $\mu$l of distilled water is added to the test tube and is mixed with the vortex for 10 sec.
3. Five ml of milk sample is added to the test tube and it is mixed with the vortex for 10 sec allowing the milk to rise and fall 10 times.
4. The test tube is incubated at 65C for 2 min.
5. A Reagent A tablet is added to the test tube.
6. The tube is vortexed for 10 sec and then incubated at 65C for 2 min.
7. The tube is centrifuged for 3 min and liquid poured off immediately afterwards.
8. The fat ring is removed and wiped dry with cotton swabs.
9. Three hundred $\mu$l of distilled water is added and the tube is vortexed to break up the sedimented pellet.
10. Three ml of scintillation fluid is added to the test tube and it is capped.
11. The tube is then counted in the analyzer for 1min.

The count per minute (cpm) is compared with a control point to decide if the sample is positive or negative. If the cpm is at or below the control point, the sample is considered positive. If it is above the control point, it is considered negative. The control point is determined by analyzing reconstituted positive control samples that are provided with the Charm II test, and adding 15% to the mean of six analyses.

# APPENDIX K

## CHARM II METHOD FOR SULFA DRUG DETERMINATION .

Materials provided with Charm II sulfa kit by Penicillin Assays Inc., Malden MA.:

| | |
|---|---|
| Reagent Sm | -($^3$H)sulfamethazine |
| Reagent R | -Binding Reagent |
| Positive Control Standard | -10 ppb sulfamethazine |

Other materials:

| | |
|---|---|
| Scintillation counter with $^3$H channel | -Charm II reader |
| Scintillation cocktail | -Packard Opti-flour |

pipettes

distilled water

test tubes

centrifuge

heating block

vortex mixer

cotton swabs

Test Procedure:

1. A Reagent R tablet is added to a test tube.
2. Three hundred $\mu$l of distilled water is added to the test tube and it is mixed with the vortex for 10 sec allowing the milk to rise and fall 10 times.
3. Five ml of milk sample is added to the test tube and it is mixed with the vortex for 10 sec.
4. The test tube is incubated at 35C for 2 min.
5. A Reagent Sm tablet is added to the test tube.
6. The tube is vortexed for 10 sec and then incubated at 35C for 2 min.
7. The tube is centrifuged for 3 min and liquid poured off immediately afterwards.
8. The fat ring is removed and wiped dry with cotton swabs.
9. Three hundred $\mu$l of distilled water is added and the tube is vortexed to break up the sedimented pellet.
10. Three ml of scintillation fluid is added to the test tube and it is capped.
11. The tube is then counted in the analyzer for 1min.

The count per minute (cpm) is compared with a control point to decide if the sample is positive or negative. If the cpm is at or below the control point, the sample is considered positive. If it is above the control point, it is considered negative. The control point is determined by analyzing reconstituted positive control samples that are provided with the Charm II test, and adding 15% to the mean of six analyses.

# APPENDIX L

## CHARM II METHOD FOR TETRACYCLINE DETERMINATION .

Materials and procedures provided with Charm II tetracycline kit by Penicillin Assays Inc., Malden MA.:

| | |
|---|---|
| Reagent T | -($^3$H)tetracycline |
| Reagent TAb | -Binding Reagent |
| Positive Control Standard | -30 ppb oxytetracycline |

Other materials:

| | |
|---|---|
| Scintillation counter with $^3$H channel | -Charm II reader |
| Scintillation cocktail | -Packard Opti-flour |
| pipettes | |
| distilled water | |
| test tubes | |
| centrifuge | |
| heating block | |
| vortex mixer | |
| cotton swabs | |

Test Procedure:

1. A Reagent TAb tablet is added to a test tube.
2. Three hundred $\mu$l of distilled water is added to the test tube and it is mixed with the vortex for 10 sec allowing the milk to rise and fall 10 times.
3. Five ml of milk sample is added to the test tube and it is mixed with the vortex for 10 sec.
4. A Reagent T tablet is added to the test tube.
5. The tube is vortexed for 10 sec and then incubated at 35C for 3 min.
6. The tube is centrifuged for 5 min and liquid poured off immediately afterwards.
7. The fat ring is removed and wiped dry with cotton swabs.
8. Three hundred $\mu$l of distilled water is added and the tube is vortexed to break up the sedimented pellet.
9. Three ml of scintillation fluid is added to the test tube and it is capped.
10. The tube is then counted in the analyzer for 1min.

The count per minute (cpm) is compared with a control point to decide if the sample is positive or negative. If the cpm is at or below the control point, the sample is considered positive. If it is above the control point, it is considered negative. The control point is determined by analyzing reconstituted positive control samples that are provided with the Charm II test, and adding 15% to the mean of six analyses.

# APPENDIX M

## DISC ASSAY METHOD OF BETA -LACTAM DETERMINATION .

Procedures are published in Standard Methods for the Examination of Dairy Products, 15th edition. (83).

| | |
|---|---|
| Agar medium | -Difco PM Indicator Agar |
| spore suspension | -Difco Bacillus stearothermophilus var. calidolactis (ATCC 10149) |
| Filter paper discs | -blank, 1.27 cm unsterile disks |
| petri plates | |
| forceps | |
| incubator | - 64 ±2C |
| waterbath | - 64C |
| Venier calipers | |

Test Procedure:

1. Reconstitute dry PM Indicator Agar as per Difco instructions with distilled water.
2. Temper to 64C.
3. Add one vial of PM thermo-spore suspension to 100 ml of agar.
4. Pour 6 ml of inoculated agar onto petri plate and let harden.
5. Place filter disc on agar and immediately add 90 $\mu$l of sample to disk with a pipette.
6. Invert the plate and incubate at 64C for approximately three to three and a half hours until well defined zones of inhibition are visible.
7. Zones are read with Vernier calipers.

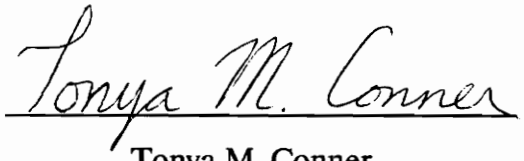
Zones of less than 14 mm are reported as no zone for inhibitory substances. A zone greater than 14 mm around a sample disc is a positive test.

# VITA

The author, Tonya Michele Conner, was born in Orange, California on February 13, 1967. She lived there, then briefly in Healdsburg, California and then in Mission Viejo, California. In 1979 her family moved to San Jacinto, California to start a wholesale nursery and that is when her agriculture experiences began. She was active in 4-H and FFA and showed dairy replacement heifers and dairy goats. Partially through these experiences she decided to attend California Polytechnic State University, San Luis Obispo, in the department of Dairy Science in the Dairy Product Technology option. While at Cal Poly she was employed by the Cal Poly Creamery and the Dairy Products Technology Center. For two summers she worked at Dannon Yogurt, Glendale, California in their quality assurance department. In her junior year, she was selected to attend Massey University, Palmerston North, New Zealand on the California State University's "International Program". She attended Massey University for a full academic year in the Food Technology department. The degree of Bachelor of Science was conferred upon Tonya in September of 1989.

In July 1990 Tonya began her studies at Virginia Tech as a candidate for the degree of Master of Science in Food Science and Technology. She has been actively involved with the Food Science Club, the Dairy Products Evaluation Team, the Graduate Student Assembly, and is the graduate student representative to the University Budget Advisory Committee.

Tonya is currently a student member of the Institute of Food Technologists and the American Dairy Science Association.



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