CONCENTRATION OF SULFAMETHAZINE IN SPRAY DRIED MILK

by

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(ABSTRACT)

A study was conducted to investigate the effect of spray drying on concentration of sulfamethazine (SMZ) in fluid milk dried to powder (<10% moisture). Antibiotic-free skim and whole (homogenized) milk were spiked at 5, 10, 50 and 100 ppb sulfamethazine levels, pasteurized and stored at 4°C till further processed. All samples were spray dried at an inlet temperature of 180 ± 2°C and outlet temperature of 100 ± 2°C and stored at -20°C until analyzed.

Sulfamethazine concentration was determined quantitatively by HPLC, a microbial receptor assay (Charm-II®) and an ELISA assay (LacTek®) and qualitatively by an ELISA method (Cite®) in milk samples before and after spray drying. Dry milk samples were reconstituted (10% w/w) for all analyses. Statistical determination of significant differences ($p = 0.05$) between fluid and dry milk samples and whole and skim milk samples was completed by paired t-tests. Sulfamethazine concentrations increased 81.4% and 84.1% in skim and whole milk respectively at 100 ppb spiked level but were lower than expected increase of 88-91% based on their total solids for whole and skim milk as obtained by

modified FDA HPLC method. At lower levels of 5 and 10 ppb, the HPLC method was not sensitive enough to provide usable data. Increase in sulfamethazine concentration from fluid to dry milk was also determined by Charm-II® and LacTek® techniques. Poor recoveries and variability in data were evident due to binding of sulfamethazine to undetermined milk components as a result of processing and storage also due to break-down of sulfamethazine (mp = 176°C) at 180°C during spray drying. Sulfamethazine 163 ppb by LacTek® and 94.6 ppb by Charm-II® (at a spiked level of 10 ppb fluid milk) was successfully removed from dried milk after 120 min using supercritical CO_2 (pressure = 5500 psi, 50°C).

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INTRODUCTION

Presence of drug residues in milk is a major concern in the United States and consumers' confidence in the dairy industry's ability to market a wholesome, uncontaminated product is in serious question. The presence of certain drug residues in milk represent a potential health hazard to consumers due to their allergenic properties (5). For example, a recent study by the National Center for Toxicological Research indicated that sulfamethazine (SMZ), a commonly used sulfa drug, may be a thyroid carcinogen (20). Sulfamethazine is rapidly gaining nationwide attention as its residues have been found in milk (5). A survey conducted by FDA in 10 U.S. cities documented the presence of sulfamethazine in 5 of 49 milk samples at concentrations above the allowable action level of 10 ppb (17). Similarly, 70% of market samples and tanker raw milk tested in the northeast U.S. were contaminated with sulfonamides at above 25 ppb and SMZ was found as the major contaminant (8).

Sulfa drugs were the first drugs in history to control systemic bacterial infections (19). They still find important application today but consumer concern about their use has increased in the past two years when drug residues in milk were detected. Sulfonamides are available "over the

counter" in agricultural stores and thus are commonly used by veterinarians and dairy farmers to prevent acute skin infections in dairy animals (8). Extra label use of sulfonamides, treatment for problems in addition to skin infections, can lead to sulfa drug contamination in milk.

Drug residues in milk should be avoided for several reasons: they are illegal; they can cause serious reactions in ultra-sensitive consumers, and some residues interfere with starter cultures used in processed milk products (5). Current concern is focused on contamination of fluid milk but contamination of concentrated dairy products with drug residues could also be a possibility. If a high solids milk product, such as dried milk, were made from a fluid milk supply with low levels of drug residues, the possibility exists that the drug residue would also be concentrated. Thus, if a fermented dairy product, such as yogurt or cottage cheese, were made with this contaminated high solids dairy product in the formulation there may be a negative effect on starter culture growth resulting in a loss of product quality and quantity. In addition, health concerns and legal issues may be a reality. Concentrations above 10 ppb SMZ, the FDA specified level of concern ("safe" level), would lead to adulteration of other products in which the contaminated concentrated milk product is used.

The objectives of this study were to:

1) investigate whether sulfamethazine is concentrated on spray drying the milk and extent to which it is concentrated. Also to determine, if it gets concentrated, whether the concentration of sulfamethazine increases above the allowable level of 10 ppb for fluid milk which would then lead to an adulteration problem of other dairy products;
2) compare sulfamethazine concentration in whole milk and skim milk after spray drying to determine how it relates to the fat components of the milk;
3) to compare if a microbial receptor assay and ELISA method could yield reliable quantitative results;
4) determine the potential of removing sulfamethazine by supercritical fluid extraction (SFE) from spray dried milk. Supercritical fluid extraction has been found to be useful in various foods, on large and small scales, but more research needs to be completed in order to understand, optimize, and apply this technology in the removal of SMZ from spray dried milk.

Hypotheses tested in relation to these objectives included: 1) sulfamethazine would increase approximately 88-92% in whole and skim milk assuming 12% and 8% total solids, respectively, equivalent to moisture removed from fluid milk, yielding a final product with SMZ concentrations

greater than the safe level; 2) fat contents would not affect extent of concentration, 3) rapid assay methods would yield reliable quantitative results and are easier and less-time consuming than extraction and HPLC methods; and 4) SMZ could be extracted from dry milk powder using supercritical carbon dioxide.

LITERATURE REVIEW

Use of antibiotics in animal husbandry has led to concern about presence of drug residues in foods derived from animal sources. These residues could cause allergic reactions, thus pose a potential health hazard to consumers. A recent study by the National Center for Toxicological Research indicated that sulfamethazine (SMZ) may be a thyroid carcinogen (20).

In addition, presence of these residues in cultured dairy products could lead to quality and economic loss of product (5). Drug residue contamination of milk may occur when lactating cows are treated for mastitis therapy, invert feeding, deliberate feeding or use of sulfa-containing boluses to prevent infection in post-calving cows (5,11). Many classes of antibiotics are used in treatment of animal diseases but few are approved for use in lactating animals (9). In many cases those drugs approved for use in lactating animals are not effective in treating mastitis (33).

It is a common practice to treat sick lactating animals with drugs approved for use in other species or at higher dosages. This is called extra-label drug use, an illegal act according to the Code of Federal Regulation (9). All drugs should have a label describing dosage, withdrawal

time, and method of medication in a particular species. Moreover, drugs with no withdrawal time should not be used because there is not adequate clinical data available to determine the time needed to eliminate drug residue from tissue or milk (33).

Extra-label use of drugs in treating lactating animals makes it difficult to detect drug presence. Many dairy farmers, knowing the Bacillus stearothermophilus disc assay is inadequate for detecting drugs other than the B-lactam class, switched to drugs that are not detected, particularly SMZ (17). The dairy industry has routinely checked for presence of penicillin but recent reports of milk contamination with other drug residues has placed pressure on the industry to prevent contaminated milk from reaching market shelves. Sixty-four percent of milk samples in the New York City area, central New Jersey, and eastern Pennsylvania contained one or more antibiotic residues; sulfonamides were the most predominant residues (5). Similar results were reported by Ingersoll (1990b). In contrast, FDA found 1.5% SMZ residue incidence in its survey of the milk supply in 23 states (18). Legislative and consumer pressure has been placed on FDA to work with state agencies, industry, and veterinarians to stop use of drug in animals immediately (39) because sulfamethazine is potentially harmful, even in small quantities.

The dairy industry has been operating on the principle of dilution. During raw milk collection, milk suspected to have low levels of contamination is diluted with large quantities of uncontaminated milk, thus resulting in no detectable residues in milk and its products (33). Surplus milk is frequently processed into various forms such as dry milk powder, evaporated or condensed milk to extend useful shelf-life. Surplus milk is dried and later used during low production periods to standardize milk and keep cheese production at a constant level year round (35). In many developing countries as well as developed countries, powdered milk is used commonly in the manufacture of ice cream, yogurt, confectionaries, and desserts (35).

Spray drying concentrates the solid matter in milk by removing moisture. SMZ is heat stable, thus if present in fluid milk it would not be destroyed at high temperatures of spray drying. We hypothesized that SMZ would be concentrated along with other water soluble non-volatile solid matter. Therefore, if milk contaminated with a low concentration of SMZ is used for spray drying and SMZ is concentrated into final product at levels above 10 ppb, then use of this powdered milk would be a potential health hazard and could result in product and economic loss.

Sulfa Drugs and Their Mode of Action

Sulfa drugs are an important class of chemotherapeutic agents and were the first agents to be effective in the treatment of bacterial diseases (19). Enzyme inhibition and pseudometabolite formation are two mechanisms by which sulfa drugs interfere in bacterial growth. Para-amino-benzoic acid (PABA), the key chemical involved in enzyme inhibition and an essential component or a building block of folic acid (1), is required for the normal growth of both bacterial and mammalian cells (19). The primary action of sulfa drug is a competitive inhibition of one or more enzymatic reactions involving PABA. They prevent the synthesis of folic acid and are toxic to those bacteria which synthesize their own folic acid vitamins (1). As a result of this mechanism, sulfa drugs do not kill bacteria but prevent the further growth and reproduction of those already present and are termed bacteriostatic (19). The relationship between the basic dissociation constant (pKa) of sulfa drugs and its bacteriostatic property is a parabolic type with a maximum effect occuring between pka 6 and 7.4 (19). The pka of SMZ is 7.4 thus it has a good bacteriostatic property.

Sulfamethazine is 4-Amino-N-(4,6-dimethyl-2-pyrimidinyl)benzenesulfonamide (Figure 1) and has a molecular weight of 278.32. Its melting point is 176°C

(178-207°C). Sulfamethazine is primarily water soluble (100 mg/100 ml water at 25°C) with a low level of solubility in alcohol. The high water solubility of SMZ favors bacteriostatic activity (19). The lipid-water partition coefficient of SMZ is 0.33 (octanol/water). Therefore, in milk, SMZ will be primarily in aqueous phase and associated with other water soluble components of milk such as lactose and proteins. SMZ appears to be stable to heat and moisture and there is no evidence of its destruction either by heat or moisture (4).

It has been shown that some sulfa drugs are teratogenic in rats. They cross the placenta and are excreted into the mother's milk (19). This is probably the mechanism by which SMZ gets into the milk of lactating dairy animals.

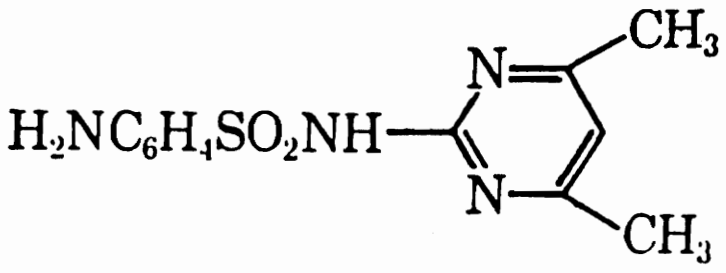

Figure 1. Structure of sulfamethazine.

Protein Binding

Protein binding is a significant factor in the action of sulfa drugs as they are acidic and bind to proteins in an inactivating but reversible process (19). N^4-acetylated derivatives are bound more strongly than its precursors According to Blanchflower et al. (1988), SMZ appears to bind to commercially pelleted feed matrix as a result of heat, pressure and moisture used in the pelleting process.

Sugar binding

The reaction of reducing sugars with sulfonamides was studied by Fleeker et al. (1985), Paulson et al. (1981) and Sheth et al. (1990). The N^4-aromatic amino group of sulfonamides react with reducing sugars to form a variety of sugar-bound compounds. Sheth et al. (1990) reported that sugar-bound sulfa compounds exhibited different chromatographic behavior than free sulfathiazole. Charm-II® did not detect sugar-bound sulfonamide or any N-substituted sulfonamide (36). Similarly, the effect of sulfonamide bound to reducing sugars in ELISA-type analyses was dramatic on its detection limit and even low levels of sugar-bound sulfonamides could affect quantitation. It was suggested that some sugar-bound sulfonamide could be released from the compound by aqueous dilution and acidification yielding a

free sulfonamide.

Giera et al. (1982) isolated a major polar metabolite, N^4-glucopyranosylsulfamethazine, from a reaction between sulfamethazine and D-glucose in swine liver and muscle tissue. Epstein et al. (1988) studied the effect of heat on residues of SMZ in muscle tissue subjected to 2-3°C and 122°C and noted a loss of 10-15% and 50% for SMZ respectively. Parks (1984) discovered transformation of SMZ to its N^4-glucopyranosyl derivative in swine liver stored at -20°C for 2 years. The derivative accounted for 96.2% and 92.2% of the depleted sulfamethazine in stored livers, thus SMZ underwent unknown reactions during frozen storage to form its derivatives. Murtha et al. (1977) reported a 10% decrease in SMZ level in calf liver stored at -20°C for 40 days.

High Pressure Liquid Chromatography

Sulfamethazine has been analyzed by different techniques using high pressure liquid chromatography (HPLC) and rapid assay methods such as microbial receptor assays and enzyme immunoassays. The FDA approved quantitative method for SMZ detection is a HPLC method.

Weber and Smedley (1989) developed the FDA approved HPLC method employing a chloroform extraction followed by liquid chromatographic determination which reliably detected

SMZ in raw, pasteurized and homogenized milk at levels as low as 5 ppb. They observed an average recovery of 80.6%, 76.3% and 73.1% at 5, 10 and 20 ppb SMZ level in milk, respectively. The trend showed a decrease in percent recovery at higher concentrations of SMZ. Parks (1982) also reported 50.8% recovery of SMZ at 0.1-0.6 ppm in swine liver using HPLC technique.

According to Long et al. (1989), SMZ can be determined and isolated from infant formula samples by blending it with octadecylsilyl derivatized silica (C-18) packing, washing with hexane and then eluting SMZ with methylene chloride. Eluate was free of interfering compounds and recovery of SMZ was 99.09%. Long et al. (1990a, 1990c) used a matrix solid phase dispersion (MSPD) method for SMZ determination in milk and pork tissue, and found that it eliminated many of the difficulties (interferences) associated with traditional isolation techniques. The average % recoveries were (73.1 ± 7.4 to 93.7 ± 2.7) using MSPD method for sulfonamide determination in milk.

Ahmed and El-Gizawy (1989) investigated the interaction of B-cyclodextrin stationary phase with some sulfonamides using HPLC for quantification. They observed sulfonamides in known synthetic mixtures were quantitative and showed complete recovery (100.15%). They also noticed minor differences in retention times due to accumulation effect

and competition of seven sulfonamides in a standard mixture on the resolution from B-cyclodextrin cavity.

Microbial Receptor Assay

This method provides specific binding sites to microbial cells for which ^{14}C or ^{3}H labeled drug competes with drug residue in the sample. Charm et al. (1988a) studied microbial receptor assay for detection of sulfamethazine (75 ppb) in milk and found that SMZ was correctly identified at a 95% success rate. Incidence of false positive was about 3% and incidence of false negative was about 1%. Bacteria greater than 10^6-10^7 organisms/ml count in milk lowered the measured activity of sulfonamide by binding to the tracer, thus making it unavailable for the assay and resulting in a false positive response. This effect was observed within 5-6 days for raw milk and over a period of weeks for pasteurized milk. No interferences with receptor assay from agricultural chemicals used with cows were observed.

Enzyme-Linked Immunosorbent Assay (ELISA)

An enzyme-linked immunosorbent assay is one in which antibodies act as a specific reagent, reacting almost exclusively with enzyme conjugate. This screening method allows rapid estimation of the concentration of

sulfamethazine in milk samples. Singh et al. (1989) demonstrated that enzyme immunoassay was a rapid, sensitive and convenient screening method for sulfamethazine in swine plasma with recoveries ranging from 97.6% to 112.5%. Fleeker and Lovett (1985) also reported ELISA as an alternative method to microbial receptor assay for the detection of SMZ in swine blood with recoveries of 66%.

Supercritical Fluid Extraction

Supercritical fluid extraction is extraction by a supercritical fluid which is a substance above its critical temperature and pressure. SFE system basically consists of a pump, extraction vessel and a collective device. The safe nontoxic fluid (gas) is vented into the atmosphere after the extraction (Fig. 2). Parameters of SFE are pressure, temperature and extraction (CO_2). At critical temperature and pressure the substance to be extracted is fully solubilized in the fluid and is carried away from the vessel by the flow of the supercritical fluid and trapped by decompression of fluid (gas) in a collective vessel.

There has been little work reported on drug residue detection, quantification and isolation by supercritical fluid extraction-supercritical fluid chromatography. Ramsey et al. (1989) demonstrated the potential for the combination of supercritical fluid extraction-supercritical

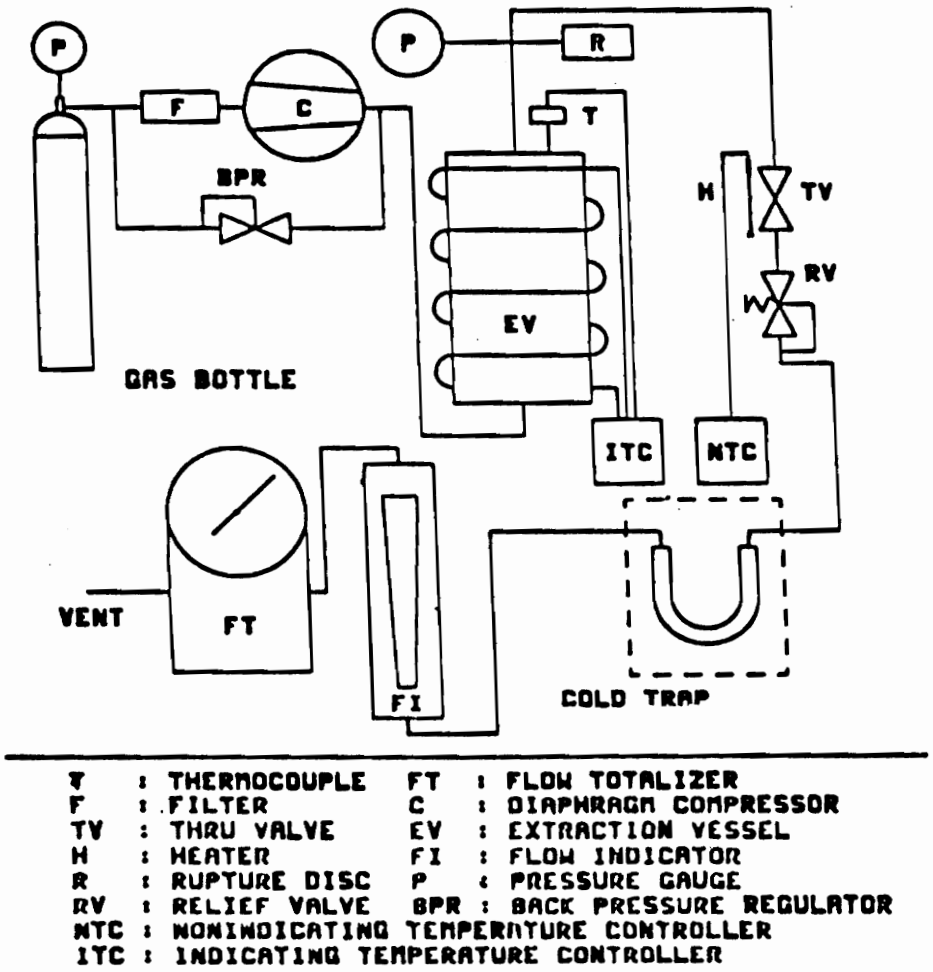

Figure 2. Schematic diagram of supercritical fluid extraction system.

fluid chromatography-mass spectrometry-mass spectrometry (SFE-SFC-MS-MS) in detection of drug residues in freeze-dried pig's kidney but observed that the sensitivity of the method was not adequate. Perkins et al. (1991) also succeeded in the identification of SMZ in spiked porcine kidney extract using SFC-MS with a moving belt interface.

MATERIALS AND METHODS

Milk Collection and Standardization

Raw milk was obtained from three first-calf heifers previously untreated with antibiotics at the Virginia Tech Dairy farm. Freshly collected milk was separated immediately using a pilot plant (Elecrem # 27093 Bonanza Industries, Inc., Calgary Alberta T2K5X3) separator and stored at 4°C for 24 hrs. A portion of milk was standardized to 3.25% fat for whole milk, retaining a supply of skim milk (0.05% fat). Standardized 3.25% fat whole milk was warmed to 38°C and homogenized at 1800 psi with an APV Gaulin two stage homogenizer (APV Gaulin Model 15 MR 15, Everett, MA., USA).

A master solution of sulfamethazine standard was prepared using 20 mg SMZ (Sigma Chemical Co. Cat, No. S-6256, Sigma Chemical Co., St. Louis, MO 63178) dissolved volumetrically in 20 ml of pasteurized skim milk. One liter of stock solution was prepared by volumetric dilution of pasteurized skim milk and thorough mixing to yield a concentration of 1 ppm. The stock solution and master solution were both stored at -20°C and thawed in water bath prior to use for each replication. Skim/whole milk samples were spiked at 5, 10, 50, and 100 pbb by diluting with skim or whole milk up to 1 volumetric liter. All samples were

mixed thoroughly.

Milk Processing

Control and spiked milk samples were batch pasteurized at 63°C for 30 min (24, 25), and immediately cooled to 15°C in ice water. Samples were then stored at 4°C (5, 38) for one to four days prior to drying.

Stored samples (4°C) were brought to room temperature of 25°C and 300 ml aliquot removed and stored at -20°C until analyses could be completed. The remaining sample was dried in a laboratory spray drier (Buchi 190 Brinkmann mini spray drier) at an inlet temperature of 180 ± 2°C and outlet temperature of 100 ± 2°C, predetermined optimum laboratory scale spray drying conditions. The spray-dried milk was cooled to room temperature (25°C) and stored in double plastic bags (24, 29) at -20°C until analyzed.

Chemical Analyses

The skim milk/whole milk controls and spiked samples were analyzed for % fat, % total solids (34) and qualitative and quantitative determination of sulfamethazine using drug detection methods such as Cite®, Charm-II®, LacTek®, and high pressure liquid chromatography (all described below) before and after spray drying.

Reconstitution (10%). The dried milk sample was weighed (10g) in a bottle and distilled water (90g) at 40°C was added to the same bottle. The bottle was capped and mixture was vigorously shaken on a vortex mixer for not less than 15 min until all solids were dissolved.

Fat Content. Babcock method for fat analysis in whole milk and cream was used to determine the percent fat and Pennsylvania modified Babcock method was used for fat determination in skim milk (34). Fat analyses were completed in duplicate on each sample and averaged.

Total Solids. Atmospheric oven method was used for determining the total solids in samples (34). Dried milk and fluid milk, three and ten grams respectively, were weighed in an aluminum dish and oven dried for 18-20 hrs. The samples were cooled in the desiccator and reweighed. Percent total solids were determined using the formula:

% TS=(WT of solids / WT of sample) X 100

All samples were completed in duplicate for total solids and averaged.

Cite®. This is an enzyme linked immunosorbent assay (ELISA) for the screening of sulfamethazine residues in bovine milk (11). This competitive immunoassay was used in

this study as a qualitative quick testing method for sulfamethazine in fluid and dried milk samples. All samples were further analyzed by HPLC, an approved confirmatory method. The Cite® Sulfamethazine Test Kit, (IDEXX Corp, 100 Fore street, Portland, MN 04101) was used for rapid detection of sulfamethazine in all samples. Results were interpreted based on comparison between the color of the control spot and the sulfamethazine spot. Dry samples were reconstituted to 10% total solids for use in this assay.

Charm II®. Charm-II® Antibody test is a rapid screening radio-immunoassay for detection and quantitation of sulfamethazine in milk. In this study, Charm-II® method was conducted on all fluid skim and whole control and spiked fluid samples as well as reconstituted (10% solids) samples of skim and whole dry milk powder. A standard curve was obtained based on standards provided by the Charm-II® Kit (Penicillin Assay, Inc., Malden, MA). Modifications of sulfamethazine competitive assay were based on recommendations from Penicillin Assays. The rinse step was eliminated and the tube was drained and wiped dry with the cotton swab. The samples were run 4-5 times and averaged.

LacTek®. Idetek's LacTek® is a competitive enzyme immunoassay (EIA) for the detection of sulfamethazine in

milk (13). Fluid milk samples and dry samples (10% reconstituted) were analyzed for sulfamethazine detection using LacTek® Sulfamethazine Milk Screening Kit (LacTek, Idetek, Inc., San Bruno, CA). SMZ standard dilutions were used to obtain a standard curve and this method used to quantitate SMZ in all experimental samples.

High pressure liquid chromatography. Determination and quantification of sulfamethazine in spiked milk samples before and after spray drying were completed by high pressure liquid chromatography (HPLC) using a modified Weber and Smedley (38) method.

A stock solution (1 mg/ml) was prepared by dissolving sulfamethazine standard (Sigma Chemical Co. Cat, No. S-6256, Sigma Chemical Co., St. Louis, MO 63178) in HPLC-grade methanol and serially diluting to the desired ppb level (5.0, 10.0, 20.0, 40.0, 100.0 ppb) with distilled water. A standard curve was obtained based on these standards.

Each milk sample (10 ml) was extracted with 50 ml of extraction solution (Chloroform-LC grade and Acetone-LC grade 2:1 v/v) in a 125 ml separatory funnel. The separatory funnel was shaken vigorously for 1 min and then vented and shaken again for 1 min. The two layers were allowed to separate for 1 min, shaken again for 1 min, vented and shaken again for another min. The two layers

were then separated for 5 min and extraction solution was separated off and filtered into a pear-shaped flask through fluted filter paper previously washed with 5 ml of extraction solution. A second extraction following the same routine, but with 25 ml of extraction solution, was completed and added to first extraction. The filter paper was rinsed twice with 5 ml of extraction solution. The combined chloroform-acetone from extractions and rinses was evaporated to dryness on rotatory evaporator at 32 ± 2°C.

To the dried pear-shaped flask, 1 ml of potassium dihydrogen phosphate (PDP) solution (0.1 M) was added and agitated vigorously for 1 min on a vortex mixer. Five ml of hexane (LC-grade) were immediately added and solution agitated again for 1 min. Layers were separated for a minimum of 15 min and the aqueous layer removed using a pasteur pipet. The aqueous layer was filtered through an Acrodisc, 0.2 um (Gelman Sciences) attached to a syringe (1 ml) into a sample vial. The sample vial was capped and sample stored at 10°C until injection. A 50 um sample was injected and the retention time for SMZ was approximately 7.0 minutes.

Analyses of standard and extracted samples were conducted utilizing a Waters HPLC, ALC Model M-6000 A (Waters Associates, Inc., Milford MASS), and an injector (Rheodyne 7125 Injector COTATI, CA.,USA) with a 50 ul loop

attached. The LC column was Phenomenex Bondclone 10 C-18, 300 X 3.9 mm (Torrance, CA), maintained at 35 ± 0.2°C. Guard column was also Phenomenex Bondclone 10 C-18, 30 X 3.9 mm (Torrance, CA). The UV detector (Waters Associates Model 440 Absorbance) was set at 254 nm for SMZ analysis, and the exit of the UV detector was connected to a Hewlett-Packard integrator (3390A Hewlett Packard Integrator). The quantity of sulfamethazine determined by UV was calculated from a standard curve prepared from varying concentrations of SMZ (5-100 ppb) (Fig.2). The solvent system was a 76:24 (v/v) ratio of KH_2PO_3 (0.1 M) (Certified ACS Fisher chemical, Fisher Scientific) to methanol (LC-grade) at an isocratic flow rate of 1.5 ml/min.

Supercritical Fluid Extraction. Sulfamethazine was extracted from dry skim milk using supercritical CO_2 (The BOC group Inc. Murray Hill, NJ) (31) with a supercritical fluid extraction (SFE) screening system (Fig 2, Super Pressure Cat # 46-19350 Newport Scientific, Inc., Jessup, Maryland) with 300 ml extraction vessel (# 41-12155). The trapping system consisted of a glass U-tube (20 cm in length and 1 cm diameter). The temperature at decompression was set at 125°C to prevent clogging of the nozzle by chilled CO_2. Extraction conditions were 50°C and 5500 psi (16). Supercritical CO_2 was passed through 10 gm sample of SMZ

spiked (10 ppb) dry milk sample for 20, 60 and 120 minutes. Dry milk (spiked) samples were analyzed before and after SFE using the LacTek®, Charm-II® and Cite® methods.

Statistical Analyses

Statistical analyses were performed using a general linear model to obtain the best fit line to determine ppb values of SMZ for HPLC, Charm-II® and LacTek techniques®.

Paired t-test was used for HPLC, Charm-II® and LacTek® to compare recovered concentrations (calculated) of SMZ in dry milk powder and fluid milk from which powder was manufactured for the 5, 10 and 100 ppb samples. Standard error of mean was used to determine variability among replications in all the samples. Significant differences among replicates for each product (skim, whole, liquid, and, dry) at each concentration (0, 5, 10, 50, 100 ppb) were determined for total solids and fat analyses.

All statistical analyses were performed using Statistical Analysis System (SAS Institute, Inc., Box 8000, Cary, NC 27511).

RESULTS AND DISCUSSION

Monitoring drug residue contamination in raw fluid milk is essential to maintaining a residue free fluid milk supply for human consumption. Further processed dairy products are not tested because it is assumed that a raw milk supply that has low or undetectable levels of residues will yield residue-free product. We explored that hypothesis by measuring sulfamethazine levels in spiked fluid milk and in milk powder manufactured from that source. The theoretical increase in SMZ concentration in dry milk as compared to fluid milk would be 88% for whole fat product and 91% for skim product based on total solids. Results are discussed based on concentration of SMZ recovered by various analytical techniques at the various concentrations (5, 10, 50, 100 ppb) at which the fluid milk was initially, spiked. Thus comparisons are made only between recovered concentrations and not between recovered and calculated spiked levels.

Analytical Detection of Sulfamethazine in Fluid and Dry Milk.

High pressure liquid chromatography. High pressure liquid chromatography was used to quantitate sulfamethazine (Fig.3) in spiked skim and whole fluid and dry milk samples

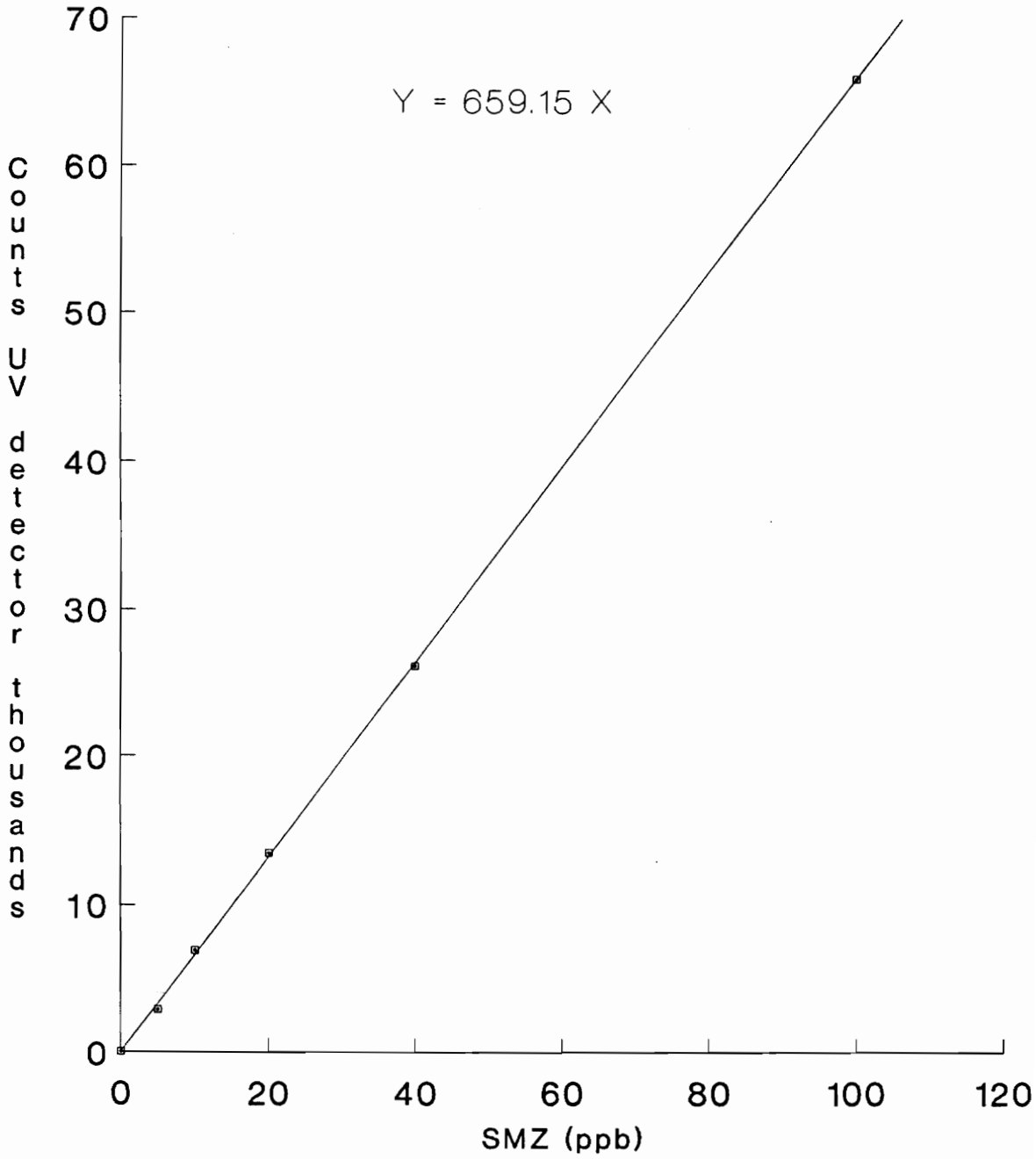

Figure 3. HPLC standard curve for sulfamethazine, using linear regression equation with r = 0.9996.

(Table I). At 100 ppb level, mean concentrations of fluid and dry milk samples were significantly different ($p=0.05$). SMZ concentration increased 81.4% and 84.1% in skim and whole milk samples, respectively, where as the expected theoretical increase was 91% and 88% for skim and whole milk, respectively. No significant difference ($p=0.05$) was observed in SMZ concentration between whole and skim fluid or whole and skim dry milk samples at 100 ppb level. The extraction method yielded mean recoveries of 52% and 75% for skim and whole fluid milk samples, respectively. Low recoveries were reported in previous work completed on SMZ determination in milk (38) and swine liver (28) by HPLC method.

Recovery of SMZ from fluid and dry samples at 5 and 10 ppb levels was highly variable and did not provide a complete set of data. The 50 ppb level was added after the second replicate because of the variability obtained at the lower levels. Recovery obtained (65% - 76%) and the percent increase (75% and 95%, skim and whole) demonstrated that, at these lower SMZ concentrations recoveries remained low and the increase in SMZ was lowered than expected in skim milk. A SMZ increase of 95% as compared to the expected increase of 88% in whole milk may be explained by variability in SMZ elution during the extraction process. A primary problem encountered with modified FDA method was poor elution

Table I. Recovery of spiked sulfamethazine from skim and whole fluid and dry milk by HPLC.

Sulfamethazine spiked concentration (ppb)	SKIM Fluid[1] (ppb Recovered)	Dry[2]	% Increase in SMZ
5.0[3]	6.7 ± 1.5	53.2 ± 32.3	14.5
10.0[4]	10.2	67.4	56.5
50.0[4]	32.5	280.0	75.0
100.0[5]	51.6[a] ± 5.3	492.9[b] ± 40.0	81.4
Sulfamethazine spiked concentration (ppb)	**WHOLE Fluid[6] (ppb Recovered)**	**Dry[2]**	**% Increase in SMZ**
5.0[4]	16.5	90.4	63.3
10.0[3]	13.0 ± 5.6	70.9 ± 14.7	114.7
50.0[4]	38.2	312.0	94.8
100.0[5]	74.6[a] ± 11.7	522.7[b] ± 30.1	84.1

[1] Pasteurized skim fluid milk.
[2] 10% reconstituted.
[3] Mean and standard error of 2 replicates for whole and skim fluid and dry milk samples. All calculations based on the total solids of fluid and dry milk.
[4] Based on single value.
[5] Mean and standard error of 3 replicates for whole and skim fluid and dry milk samples. All calculations based on the total solids of fluid and dry milk.
[6] Homogenized, pasteurized whole fluid milk.
[a,b] Means at same concentration levels within columns and rows with different letters are significantly different (p=0.05) based on paired t-test analyses.

behavior of the extracted analyte at 10 ppb and below (38). The general trend of this research showed an overall increase in concentration of SMZ from fluid to dry milk at all levels.

Figure 4 shows typical chromatograms of a 20 ppb SMZ standard (retention time (RT) = 7.22 min), an unspiked control fluid milk sample and fluid whole milk spiked at 100 ppb (RT = 7.47 min) at a flow rate of 1.5 ml/min. The modified FDA HPLC method yielded an easily identifiable sulfamethazine peak. The control sample clearly demonstrated the absence of a peak at 7.47 min, ignoring baseline noise which was due to electronic disturbance in our integrator.

Microbial receptor assay (Charm-II). Charm-II® analyses for SMZ in skim and whole fluid and dry milk samples were much easier and less time consuming than HPLC method. At 5 ppb spiked levels no significant differences ($p=0.05$) in fluid and dry milk samples were found due to high variability in results but significant differences were obtained at 10 and 100 ppb spiked levels. There was no significant difference in skim and whole milk at each concentration level (Table X-XII, see appendix). An increase of 60-120% in SMZ concentration from fluid to dry milk powder in skim and whole milk samples was obtained (Table II).

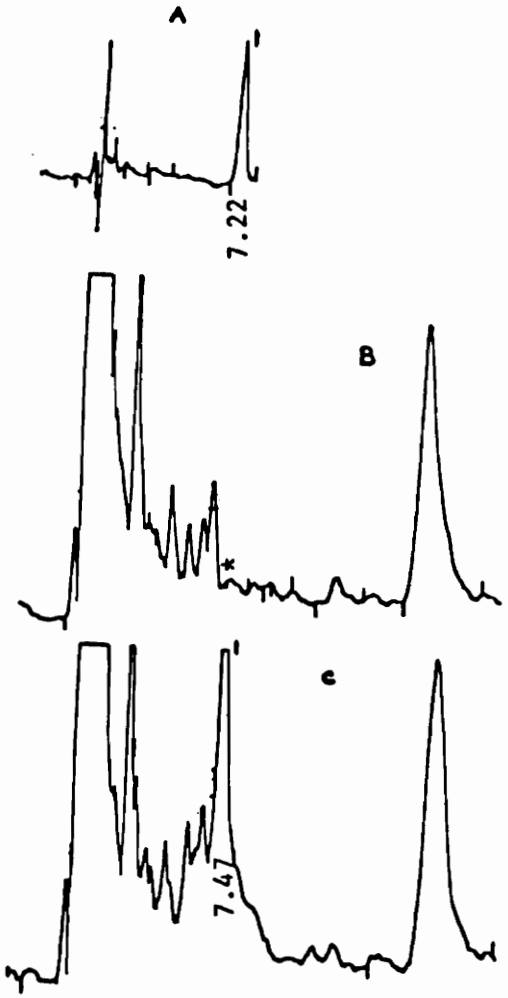

Figure 4. HPLC chromatograms of: A, 20 ppb SMZ standard (Retention time (RT) = 7.22 min); B, control (* = no peak); C, spiked (100 ppb) milk (RT = 7.47 min). Peak 1 = SMZ.

Table II. Recovery of spiked sulfamethazine from whole and skim fluid and dry milk by Charm-II®.

Sulfamethazine spiked concentration (ppb)	SKIM Fluid[1] (ppb Recovered)	Dry[2]	% Increase in SMZ
5.0[3]	3.2[a]± 3.1	28.2[b]± 25.0	97.8
10.0[5]	9.9[a]± 4.8	94.9[b]± 46.6	68.4
50.0[4]	52.8	613.1	101.0
100.0[5]	70.0[a]± 47.0	805.8[b]± 422.8	120.2
Sulfamethazine spiked concentration (PPb)	**WHOLE Fluid[6] (PPb Recoverd)**	**Dry[2]**	**% Increase in SMZ**
5.0[3]	3.2[a]± 1.5	21.7[b]± 16.6	60.1
10.0[5]	6.2[a]± 3.5	65.2[b]± 32.8	107.4
50.0[4]	18.5	152.2	95.3
100.0[5]	23.7[a]± 9.7	188.0[b]± 89.6	71.5

[1] Pasteurized skim fluid milk.
[2] 10% reconstituted.
[3] Mean and standard error of 2 replicates for whole and skim fluid and dry milk samples. All calculations based on the total solids of liquid and dry milk.
[4] Based on single value.
[5] Mean and standard error of 3 replicates for whole and skim fluid and dry milk samples. All calculations based on the total solids of fluid and dry milk.
[6] Homogenized, pasteurized whole fluid milk.
[a,b] Means at same concentration levels within columns and rows with different letters are significantly different (p=0.05) based on paired t-test analyses.

Increased SMZ concentrations greater than expected may be a result of lower recovery for fluid milk and a better recovery for skim milk thus yielding a greater calculated increase. Results were still highly variable and could be attributed to the non-linear nature of the standard curve (Fig.5) above 20 ppb or to a lack of sensitivity of this assay at high SMZ concentration. Recoveries at 5 and 10 ppb ranged from 64-99% as the standard curve is linear below 10 ppb. At the 50 and 100 ppb levels, the recoveries were 105% and 70%, respectively, in skim fluid milk samples but only 37.5% and 47.5% respectively in whole milk. It appeared that the higher fat content in the whole milk may have interfered with the sensitivity of the assay at 100 ppb. The presence of inactive metabolites at higher SMZ concentrations may have increased variability as these do not bind to receptor sites in cells and are not detected by the receptor assay method (7).

As with the HPLC method of evaluation, results from the Charm-II® analyses documented an increase in SMZ concentration in dry milk powder to concentrations 6 to 10 times more than that found in fluid milk.

Quantitative ELISA Method (LacTek®). The LacTek® method was equivalent in ease of use and time requirements to the Charm-II® method and easier than the HPLC method.

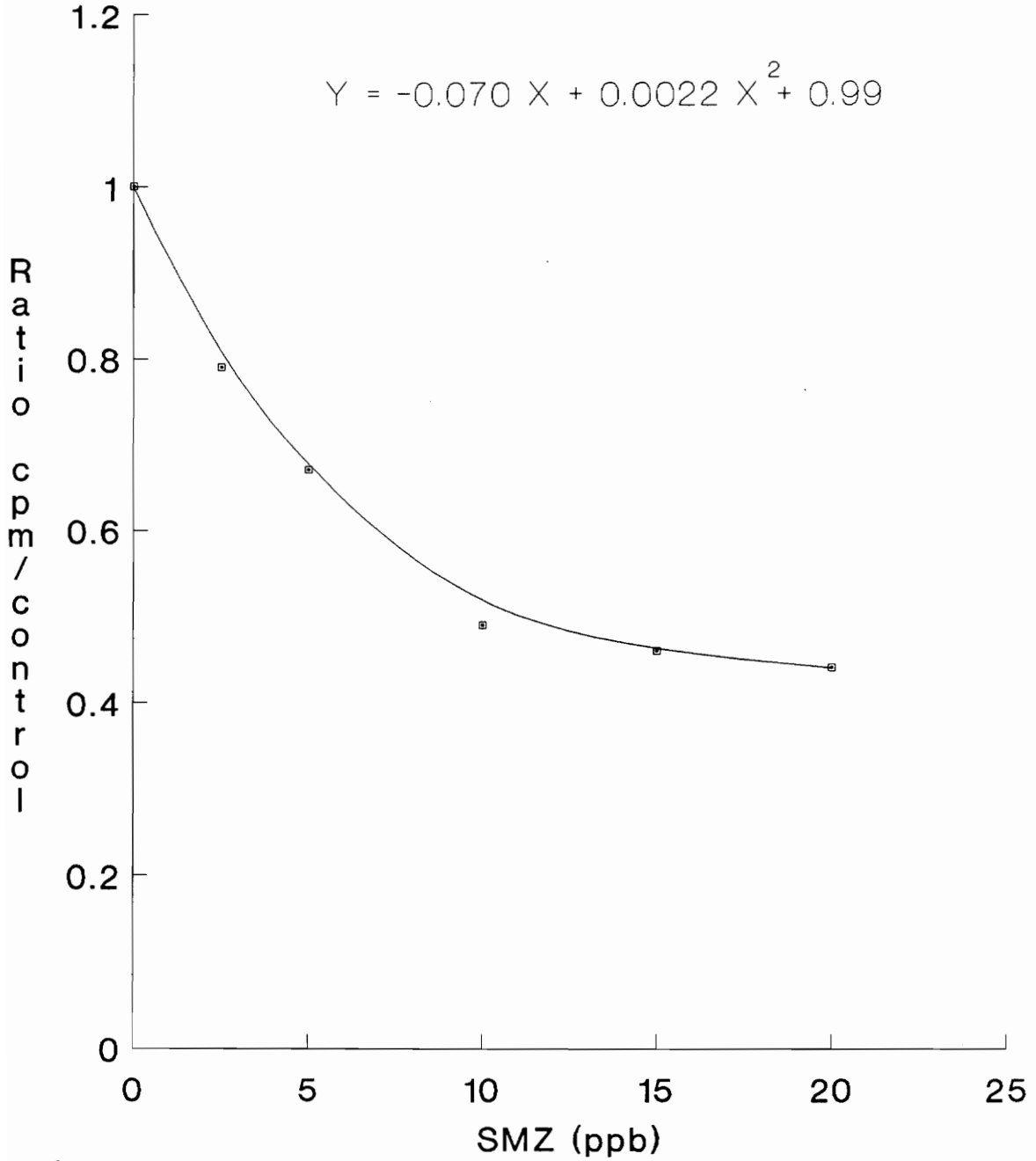

Figure 5. Charm-II® standard curve for sulfamethazine with r = 0.9937.

Variability in data was also a problem with the LacTek® method. Percent increase in SMZ from liquid to dry milk is presented in Table III. A standard curve (Fig.6) of ratio (a/b; a = absorbance of sample, b = absorbance of control) versus concentration of SMZ was obtained for quantitation of SMZ. At 5 and 10 ppb levels, SMZ concentrated 50-122% respectively in whole milk and 80-89% in skim milk. At higher concentrations, results were not very consistent due to the type of assay and its relative lack of sensitivity. Again a significant increase ($p=0.05$) in mean SMZ concentration in dry milk as compared to fluid milk at 5, 10 and 100 ppb levels was found. There was no significant difference between concentration of SMZ in skim and whole fluid nor between skim and whole dry milk SMZ concentrates at each level. Milk, being a complex mixture, might react in a non-specific manner with the reagent to cause variability in the assay (11). The concentrations recovered were higher than those spiked in the milk, suggesting another component in the milk may also be competing for active sites. Another possibility could be the breakdown of SMZ (mp = 176°C) at 180°C of spray drying and this breakdown product might have also competed for the active sites.

Variability in recoveries may be attributed to the complexity of the milk system and breakdown of sulfamethazine at >176°C. Researchers using different

Table III. Recovery of spiked sulfamethazine from skim and whole fluid and dry milk by LacTek®.

Sulfamethazine spiked concentration (ppb)	SKIM Fluid[1] (ppb Recovered)	Dry[2]	% Increase in SMZ
5.0[3]	9.6[a]± 3.1	91.4[b]± 3.7	88.4
10.0[5]	18.2[a]± 1.5	162.8[b]± 27.9	79.2
50.0[4]	87.8	774.6	76.6
100.0[5]	103.5[a]± 45.9	1229.4[b]± 93.4	151.1
Sulfamethazine spiked concentration (ppb)	**WHOLE Fluid[6] (ppb Recovered)**	**Dry[2]**	**% Increase in SMZ**
5.0[3]	8.5[a]± 2.1	41.8[b]± 21.0	49.8
10.0[5]	14.7[a]± 2.4	147.1[b]± 44.3	122.0
50.0[4]	45.8	652.6	165.0
100.0[5]	70.4[a]± 20.4	624.4[b]± 168.7	100.1

[1] Pasteurized skim fluid milk.
[2] 10% reconstituted.
[3] Mean and standard error of 2 replicates for whole and skim fluid and dry milk samples. All calculations based on the total solids of fluid and dry milk.
[4] Based on single value.
[5] Mean and standard error of 3 replicates for whole and skim fluid and dry milk samples. All calculations based on the total solids of fluid and dry milk.
[6] Homogenized, pasteurized whole liquid milk.
[a,b] Means at same concentration levels within columns and rows with different letters are significantly different (p=0.05) based on paired t-test analyses.

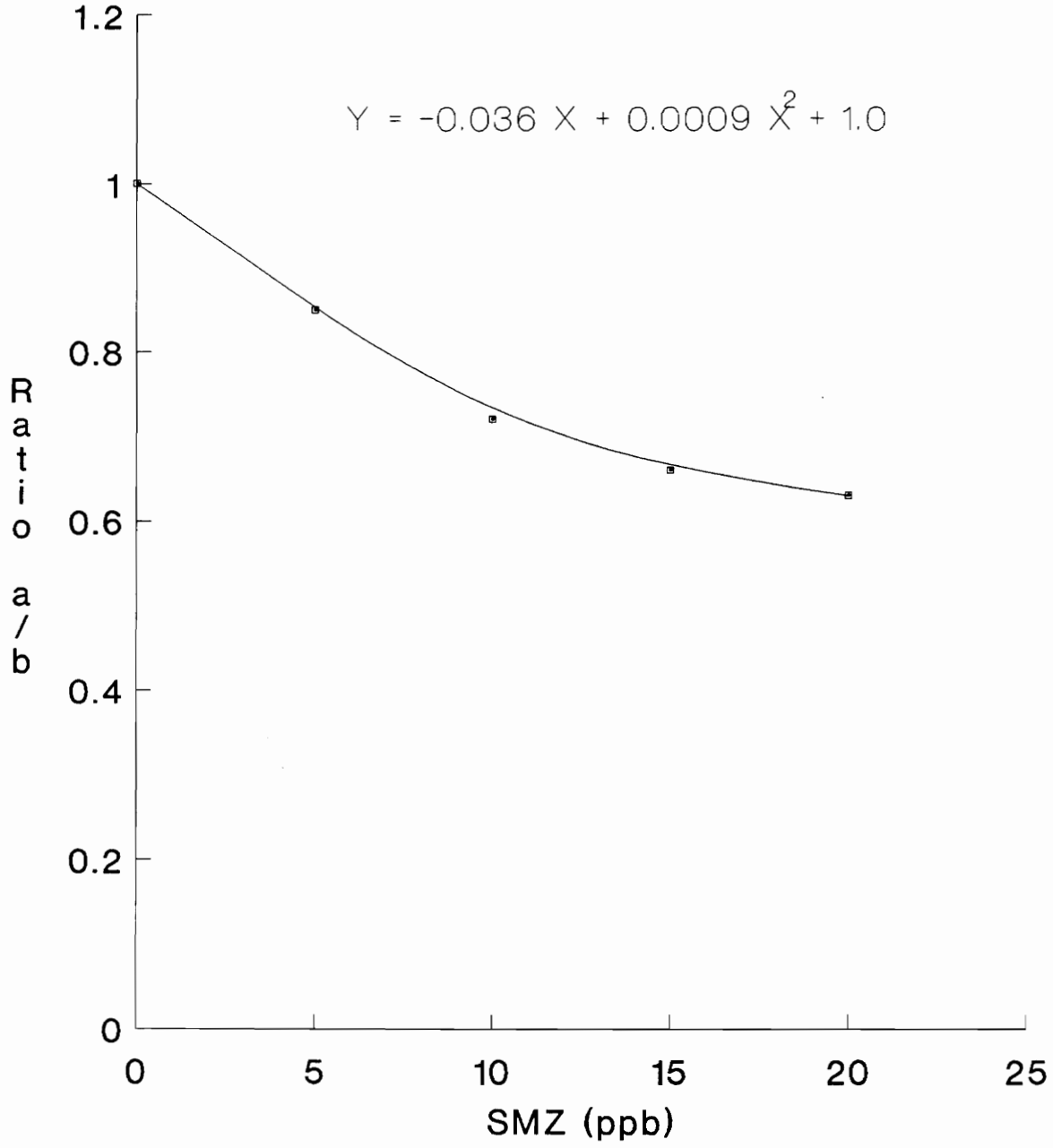

Figure 6. LacTek® standard curve for sulfamethazine with r = 0.99935.

biological systems have observed some variability in assessing SMZ using enzyme immunoassay methods. Singh et al. (1989) also observed recoveries of 97.6 to 112.5% with mean recovery of 103% using enzyme immunoassay (EIA) method for determination of SMZ in swine plasma. In contrast, 62-73% recoveries were observed in a study using enzyme immunoassay for screening of sulfamethazine and its metabolites in swine blood (0.01ppm) (13). Discrepancy of recovery among different types of test methods could be explained by differences in sensitivity and detection time of each test and inhibitors present other than SMZ. In a previous study discrepancy of positive and negative results of two different tests was observed (6).

Each of these methods (HPLC, Charm-II®, LacTek®) has demonstrated that SMZ in fluid milk is concentrated into dry milk. The percent increase using HPLC is in the range of 75-94% for skim and whole milk at 50 ppb and 81.4-84.1% at 100 ppb using HPLC. For Charm-II® the increase was 97.8% and 60.1% for skim and whole milk at 5 ppb and 68.4% and 107.4% at 10 ppb. Using LacTek® the increase was 88.4% and 49.8% for skim and whole milk at 5 ppb and 79.2% and 122.0% at 10 ppb level, at >50 ppb level the percent increase is >100%. The expected increases in SMZ concentration on spray drying based on total solids of milk were 88% and 91% for whole and skim milk. Recoveries lower than the expected

values were probably due to the binding effect of SMZ with milk components and also the high temperature of processing (180°C) and breakdown of SMZ at such high temperature. Values greater than the expected increase were possibly due to some component of milk or the breakdown products of SMZ competing for the active sites in rapid screening assays. Sulfamethazine levels below or at the safe concentration level for SMZ, as established by FDA, in fluid milk will increase to levels greater than the safe level if that fluid milk is dried.

Qualitative ELISA Method (Cite®). This is a polyclonal antibody-based assay for detection of SMZ and its major metabolites in fluid milk. This test was used as a rapid subjective determination of presence of SMZ prior to HPLC analyses (Table IV). At 5 ppb the test was negative for all 3 replications of whole and skim fluid and dry milk samples. At 10 ppb Cite® was inconclusive for both fluid and dry (10% reconstituted) whole and skim milk. The test was clearly positive for 50 and 100 ppb samples in all 3 replications and for all milk types. In contrast to the results obtained at 5 and 10 ppb levels, Bishop et al. (1991) reported positive Cite® results at 5 ppb for raw whole milk samples.

Table IV. Detection of sulfamethazine in fluid[1] and reconstituted dry[2] milk by Cite®.

Sulfamethazine (ppb)	Rep 1	Rep 2	Rep 3
0	-	-	-
5	-	-	-
10	±	±	±
50	+	+	+
100	+	+	+

[1] Whole and skim milk.
[2] 10% reconstituted whole and skim milk.

Effect of Freezing and Heating on SMZ Recovery

The conflicting results obtained for Cite® analyses in this study as compared to those reported by Bishop et al. (1991), lower recoveries than expected and variability in results obtained by HPLC, Charm-II®, and LacTek® methods suggested that some type of interference may be responsible. Sheth et al. (1990) reported that heat caused sulfathiazole to bind to reducing sugar and inhibited detection of the compound without altering the primary structure. A brief study was undertaken to determine if heating, as would occur during pasteurization (63°C, 30 min) and spray-drying (inlet temperature of 180 ± 2°C, outlet temperature of 100 ± 2°C) of spiked fluid milk samples, or freezing (- 20°C from 1-3 months), the storage method used to retain product quality through out the study, would affect SMZ recovery (Table V). LacTek® results demonstrated a decrease in recovery from 20.2 ppb in untreated milk to 16.7 ppb in pasteurized milk with a further decrease in recovery to 4.1 ppb from a frozen and thawed pasteurized milk at 10 ppb spiked level. Similar trends were observed using HPLC at 40 and 100 ppb levels. The binding of SMZ to proteins and/or reducing sugar present in milk samples upon heating is a possible explanation for the variability in recovery observed in this study. Sheth et al. (1990) demonstrated the reaction of the N^4 aromatic group of sulfonamide with reducing sugar to form sugar-

Table V. Effect of pasteurization and freezing on recovery of spiked sulfamethazine from raw whole milk by HPLC and LacTek®.

Method	Spiked SMZ (ppb)	Process Untreated (ppb Recovered)[2]	Pasteurization	Freezing[1]
HPLC	40	27.5	18.5	nd[3]
	100	93.3	59.0	nd
LacTek[4]	10	20.2	16.7	4.1
	100	57.6	65.7	51.5

[1] Samples frozen for one week at -20^0 C.
[2] Based on single replication.
[3] nd, no data available.
[4] All calculations based on control samples.

sulfonamide complex. Blanchflower et al. (1988) also suspected the cause of their poor recoveries to be irreversible binding of SMZ to feed matrix. Similar results were reported by Epstein (1988), Giera et al. (1982) and Parks (1984) for effect of heating sulfamethazine residues in meat. Sheth et al. (1990) observed that higher heating temperature also increased the rate of irreversibly bound glucose to sulfonamide. The spray drying temperature of 180°C used in this study was above the melting point of SMZ (mp = 176°C), thus it is possible that at this high temperature some lactose was irreversibly bound to SMZ and SMZ was not extracted out during analytical procedure which would have lead to a decrease in recovery.

The complete study was conducted over a four month period during which fluid and dry milk samples were frozen to retain high quality until all analyses for three replications could be completed. Samples from the first, second and third replicates were stored at -20°C for three, two, and one month periods. Charm-II® and LacTek® analyses were completed on all samples of all replicates at one time. This storage factor may have also contributed to variability in results as demonstrated by this study. Storage at -20°C decreased concentration of SMZ minor residues in swine muscle and liver after only 15 days by 13.9% and 12.6% respectively (10). A 10% decrease in SMZ concentration in

calf livers was found after 40 days of storage at -20°C (Murtha et al. 1977). Parks (1982) reported that liver tissue, in which SMZ concentration was reduced after freezing, had high levels of N^4-glucopyranosylsulfamethazine, accounting for over 90% of the depleted SMZ residues.

The evidence by Sheth et al. (1990) suggests that binding by reducing sugar is not permanent but that free, active sulfonamides could be released from the complex by acidification or aqueous dilution. The dilution of the dry powder for analyses may have permitted some lactose-bound SMZ to be released, providing another possible explanation for variability in recoveries. It is evident that heating and freezing of SMZ-contaminated milk samples can affect recovery of SMZ.

Comparison Of Three Quantitation Methods used in This Study

Each of the three methods used for quantitation of SMZ had advantages and disadvantages as compared to the other methods. HPLC method was not sensitive at lower levels (5 and 10 ppb) of SMZ detection. A probable reason could be the extraction method which was not effective enough to extract SMZ out of milk as a result of binding. At higher levels there was enough SMZ extracted out to be detected and give a proper SMZ peak. The standard curve with a

correlation value of r = 0.9996 was used to determine the actual ppb obtained.

Charm-II® method has a limitation of non-linear nature of the standard curve (r = 0.9937) beyond 20 ppb. Therefore higher concentration of SMZ in milk were not quantified satisfactorily but at lower levels the results were satisfactory. Another disadvantage of Charm-II® is that the metabolites of SMZ interfere with the assay. Frozen storage of samples interfered with results obtained by Charm-II® method. Charm-II® is a rapid, sensitive method for raw fresh milk samples at lower levels of detection.

LacTek® is a rapid and simple technique but detection of SMZ at level < 5 ppb is questionable and the nature of the curve is also non-linear above 20 ppb. Thus at very low and at higher levels the results are not very reliable. The valid range for quantitative analysis appears to be limited to 5-20 ppb. A standard curve with a good correlation value of r = 0.99935 was used for determining the equivalent ppb in milk samples.

Extraction Of Sulfamethazine by Supercritical CO_2

Supercritical fluid extraction (SFE) has been used for industrial scale separation and isolation of a variety of compounds in various systems. The results of this study demonstrated an increase in concentration of SMZ on spray

drying the spiked liquid milk, thus it was important to determine the potential of supercritical fluid extraction for the removal of SMZ from contaminated dry milk powder. Ramsey et al. (1989) found considerable potential for SFE in separation and isolation of trace levels of contaminants in foods. Complete removal (97 - 100%) of SMZ from dry skim milk powder contaminated with SMZ at 163 ppb based on LacTek® analyses and 94.6 ppb by Charm-II®, as manufactured from fluid milk spiked at 10 ppb SMZ, was possible using supercritical CO_2 at 120 min of processing (Table VI). This result is based on the assumption that LacTek® standard curve is effective in the range of 0-5 ppb. LacTek®, Charm-II®, and Cite® analyses were completed on the SMZ-extracted sample remaining in the extraction vessel. For Cite® analyses, the analyte eluted from the SFE extraction was mixed with 10-15 drops of raw skim milk and analyzed for presence of SMZ in extract after extraction of 20, 60 and 120 minutes. Cite® analyses were positive for SMZ at each extraction times. Cite® analyses on the extraction vessel sample were negative for SMZ at each extraction. A reduction in SMZ concentration was observed even after 20 minutes but concentration of SMZ in dry powder did not fall below a safe concentration level of 10 ppb until extraction was completed for more than 60 minutes. This study then demonstrates that SMZ can be extracted from dry milk powder

Table VI. Extraction of sulfamethazine (10 ppb level)[a] from skim milk powder by supercritical CO_2[b] and detection using LacTek®[c], Charm-II®[c] and Cite®.

Minutes	LacTek® (% Extracted)	Charm-II® (% Extracted)
0	0	0
20	7	56
60	55	77
120	100	97

Minutes	Extraction vessel (Cite®)	Final extract (Cite®)
0	±	na
20	-	+
60	-	+
120	-	+

[a] Calculated concentration of SMZ in dried powder prior to SFE was 163 ppb by LacTek® and 94.6 ppb by Charm-II®.
[b] Analyses completed on sample from extraction vessel at (5500 Psi, 50° C).
[c] 10% reconstituted.
[na] Not applicable.

using supercritical CO_2.

CONCLUSIONS

Drug residue contamination of fluid milk supply has received a great deal of attention in the past few years. Sulfamethazine contamination has received most of attention because of potential health hazards and product and economic loss.

Sulfamethazine concentration in dry milk powder is of concern even if powder is manufactured from fluid milk contaminated at concentration less than regulatory level of concern. This study has illustrated that, with spray drying, sulfamethazine concentration increased 81.4% and 84.1% in skim and whole milk at a spiked concentration of 100 ppb level respectively, using HPLC analyses. This was lower than the expected increase of 88-91% for whole and skim milk. At 100 ppb level, mean SMZ concentration of fluid and dry milk samples were significantly different ($p = 0.05$). SMZ recoveries of 52% and 75% for skim and whole fluid milk, respectively, were obtained by modified FDA HPLC method. Fluid milk with SMZ concentrations as high as 100 ppb would be rejected at the manufacturing site and would not receive further processing. This level of concentration was used in this study to demonstrate that change in concentration does occur with further processing. The same change due to processing would probably occur at lower SMZ

concentrations but is not as easy to demonstrate using this analytical method. At lower spiked concentrations of 5 and 10 ppb SMZ, poor elution behavior of the extracted analyte decreased recovery. Charm-II®, LacTek® and HPLC were used as quantitative tool but Charm-II® and LacTek® were definitely much easier and less time consuming techniques than HPLC. Results were highly variable especially at higher levels due to the non-linear nature of their standard curves and the decreased sensitivity of the tests at higher concentration. Moreover, at < 5 ppb the ability of LacTek® to quantitate is questionable.

This study was conducted over a four month period and all samples were frozen to maintain high quality. This storage factor may have also resulted in variability of results with Charm-II® and LacTek®. Recoveries were higher in skim milk compared to whole milk samples at 50 and 100 ppb level using Charm-II®. It appeared that the higher fat content in whole milk interfered with the sensitivity of the assay. Cite® test was used for qualitative determination of SMZ in all fluid and dry samples (10% reconstituted).

Results were negative for SMZ at 5 ppb, inconclusive at 10 ppb and positive for 50 and 100 ppb in both skim and whole milk samples.

Due to the low recoveries and high variability in results, a study determining effect of freezing and

pasteurization on recovery of SMZ was conducted. Results demonstrated a decrease in recovery from 20.2 ppb in untreated milk to 16.7 ppb in pasteurized milk with a further decrease in recovery to 4.1 ppb from a frozen sample (thawed) at 10 ppb spiked level using Lactek® method. Similar results were obtained using HPLC (40 and 100 ppb). The results from this study provided evidence that heating at pasteurization temperatures and freezing at -20°C does reduce SMZ recovery, perhaps by binding of SMZ to undetermined milk components such as proteins or lactose. The high temperature (180°C) used for evaporation of moisture from fluid product may have melted SMZ (mp = 176°C) resulting in an undetectable analyte which may have contributed to lower recoveries.

Each of these methods (HPLC, Charm-II®, LacTek®) has demonstrated that SMZ in fluid milk is concentrated into higher levels in dry milk. Sulfamethazine levels below the established FDA safe level (10 ppb) in fluid milk will increase to levels greater than the safe level if that fluid milk is dried.

As the results of this study demonstrated an increase in concentration of SMZ on spray drying it was important to determine the potential of supercritical fluid extraction (SFE) for the removal of SMZ from contaminated dry milk. Complete removal (97-100%) of SMZ from dry skim milk 163 ppb

based on LacTek® analyses and 94.6 ppb by Charm-II® (at a spiked level of 10 ppb fluid milk) was possible using supercritical CO_2 at 120 min of processing and analyzing by Charm-II® and also assuming that standard curve is effective in the range of 0-5 ppb when analyzed by LacTek®.

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APPENDIX

Table VII. Mean and standard error of total solids (%) in whole and skim dry and fluid milk samples.

SMZ level (5 ppb)	N	Mean (%)	Standard error
LW	4	11.059	0.271
DW	4	94.658	0.036
LS	4	8.429	0.066
DS	4	92.175	0.303
(10 ppb)			
LW	6	11.332	0.174
DW	6	96.107	0.543
LS	6	8.496	0.095
DS	6	94.047	0.850
(50 ppb)			
LW	2	11.580	0.010
DW	2	97.775	0.495
LS	2	8.680	0.010
DS	2	97.210	0.040
(100 ppb)			
LW	6	11.220	0.084
DW	6	96.018	0.615
LS	6	8.496	0.066
DS	6	93.612	0.881

^{L,W} Fluid, whole.
^{D,W} Dry, whole.
^{L,S} Fluid, skim.
^{D,S} Dry, skim.

Table VIII. Mean and standard error of fat level (%) in whole and skim dry and fluid milk samples.

SMZ level (5 ppb)	N	Mean (%)	Standard error
LW	4	3.222	0.016
DW	4	2.725	0.043
LS	4	0.006	0.002
DS	4	0.007	0.003
(10 ppb)			
LW	6	3.207	0.022
DW	6	2.735	0.038
LS	6	0.021	0.009
DS	6	0.007	0.002
(50 ppb)			
LW	2	3.200	0.00
DW	2	2.75	0.00
LS	2	0.015	0.00
DS	2	0.018	0.00
(100 ppb)			
LW	6	2.982	0.012
DW	6	2.613	0.038
LS	6	0.024	0.009
DS	6	0.010	0.004

^{L,W} Fluid, whole.
^{D,W} Dry, whole.
^{L,S} Fluid, skim.
^{D,S} Dry, skim.

Table IX. T-test (p=0.05) for fluid and dry skim and whole milk at 100 ppb using HPLC.

T-test	Whole		Skim	
	Fluid (100 ppb)	Dry	Fluid (100 ppb)	Dry
N	3	3	3	3
Mean	74.630	522.693	51.605	492.605
Standard error	11.683	30.136	5.314	39.989
[A] T-value	13.863		0.940	
DF	4.0		4.0	
PROB>|T|	0.0002		0.0004	
[B] T-value	-1.7939			
DF	4.0			
PROB>|T|	0.1473			

[A] Comparing fluid and dry milk samples of each type with equal variance.
[B] Comparing skim and whole milk samples with equal variance.

Table X. T-test (p=0.05) for fluid and dry skim and whole milk using Charm-II®, at 5 ppb level.

T-test	Whole		Skim	
	Fluid (5 ppb)	Dry	Fluid (5 ppb)	Dry
N	2	2	2	2
Mean	3.215	21.695	3.220	28.195
Standard error	1.495	16.625	3.060	25.905
[A] T-value	1.1071		0.9574	
DF	2.0		2.0	
PROB>|T|	0.3836		0.4394	
[B] T-value	0.0020			
DF	2.0			
PROB>|T|	0.999			

[A] Comparing fluid and dry milk samples of each type with equal variance.
[B] Comparing skim and whole milk samples with equal variance.

Table XI. T-test (p=0.05) for fluid and dry, skim and whole milk using Charm-II®, at 10 ppb level.

T-test	Whole		Skim	
	Fluid (10 ppb)	Dry	Fluid (10 ppb)	Dry
N	3	3	3	3
Mean	6.147	65.213	9.893	94.847
Standard error	3.541	32.786	4.763	44.625
[A] T-value	1.7912		1.8126	
DF	4.0		4.0	
PROB> |T|	0.148		0.144	
[B] T-value	0.6313			
DF	4.0			
PROB> |T|	0..562			

[A] Comparing fluid and dry milk samples of each type with equal variance.
[B] Comparing skim and whole milk samples with equal variance.

Table XII. T-test (p=0.05) for fluid and dry skim and whole milk using Charm-II®, at 100 ppb level[c].

T-test	Whole		Skim	
	Fluid (100 ppb)	Dry	Fluid (100 ppb)	Dry
N	3	3	3	3
Mean	23.670	187.990	70.050	805.807
Standard error	9.742	89.566	46.955	422.749
[A] T-value	1.824		1.730	
DF	4.0		4.0	
PROB> |T|	0.142		0.159	
[B] T-value	0.970			
DF	4.0			
PROB> |T|	0.388			

[A] Comparing fluid and dry milk samples of each type with equal variance.
[B] Comparing skim and whole milk samples with equal variance.
[C] Statistical analysis on 50 ppb could not be done as it was only one replication.
[N] Number of replications.

Table XIII. T-test (p=0.05) for fluid and dry skim and whole milk using LacTek®, at 5 ppb level.

T-test	Whole		Skim	
	Fluid	Dry (5 ppb)	Fluid	Dry (5 ppb)
N	2	2	2	2
Mean	8.550	41.765	9.605	91.380
Standard error	2.100	21.035	3.155	3.650
[A] T-value	1.571		16.950	
DF	2.0		2.0	
PROB> |T|	0.251		0.004	
[B] T-value	0.278			
DF	2.0			
PROB> |T|	0.807			

[A] Comparing fluid and dry milk samples of each type with equal variance.
[B] Comparing skim and whole milk samples with equal variance.
[N] Number of replications.

Table XIV. T-test (p=0.05) for fluid and dry skim and whole milk using LacTek®, at 10 ppb level.

T-test	Whole		Skim	
	Fluid (10 ppb)	Dry	Fluid (10 ppb)	Dry
N	3	3	3	3
Mean	14.750	147.143	18.207	162.767
Standard error	2.397	44.259	1.497	27.917
[A] T-value	2.987		5.171	
DF	4.0		4.0	
PROB> |T|	0.041		0.007	
[B] T-value	1.223			
DF	4.0			
PROB> |T|	0.289			

[A] Comparing fluid and dry milk samples of each type with equal variance.
[B] Comparing skim and whole milk samples with equal variance.
[N] Number of replications.

Table XV. T-test (p=0.05) for fluid and dry skim and whole milk using LacTek®, at 100 ppb level[c].

T-test	Whole		Skim	
	Fluid (100 ppb)	Dry	Fluid (100 ppb)	Dry
N	3	3	3	3
Mean	70.413	624.367	103.483	1229.400
Standard error	20.448	168.750	45.952	93.391
[A] T-value	3.259		10.817	
DF	4.0		4.0	
PROB> |T|	0.031		0.000	
[B] T-value	0.658			
DF	4.0			
PROB> |T|	0.547			

[A] Comparing fluid and dry milk samples of each type with equal variance.
[B] Comparing skim and whole milk samples with equal variance.
[C] Statistical analysis on 50 ppb couldnot be done as it was only one replication.
[N] Number of replications.

Table XVI. Alternative FDA[1] high pressure liquid chromatography (HPLC) results from an independent laboratory.

Sample	(ppb received)
10 LW[2]	7.11 ± 1.05
10 DW[2]	5.56 ± 1.13
100 LW[2]	50.80 ± 0.45
100 DW[3]	75.30

[1] HPLC conditions: sample size; 100ul, solvent; PO_4+ 12% methanol, detector; uv-vis at 265nm absorbance, flow; isocratic, retention time of SMZ 19-26 minutes, column length; 15 cm, packing; ultra carbon and C-18.

[2] mean and standard error of 3 individuals extractions of same samples.

[3] Based on one extraction.

Figure 7. Calculation method for concentration of sulfamethazine in dry milk.

% Total solid of fluid milk = A (gm/100ml)

% Total solid of dry milk = B (gm/100ml)

Therefore 10 gm of dry milk = B/10= C

10% reconstituted milk = D = C/100ml

ppb in recontituted milk = X

Calculated ppb in reconstituted milk based on total solid of fluid and dry milk = E

$E = A/D \times X$

ppb recovered from spiked fluid milk = Y

Therefore % recovery from spiked dried reconstituted milk, Z

$Z = E/Y \times 100$

Concentration in dry milk = Z/A.

VITA

The author, Shahana Malik was born November 6th in Murree, Pakistan. She grew up in Sargodha (Pakistan) until grade 9, after which she proceeded to Nigeria with her family. She graduated with a major in Food Science and Technology from Federal University of Technology Owerri, Nigeria in July 1986. From 1986-1989 she worked with Malik Food Industry Ltd, Lahore and Milk Pak Ltd Lahore, Pakistan.

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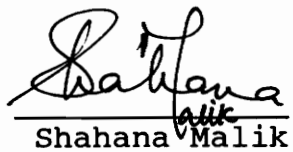

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