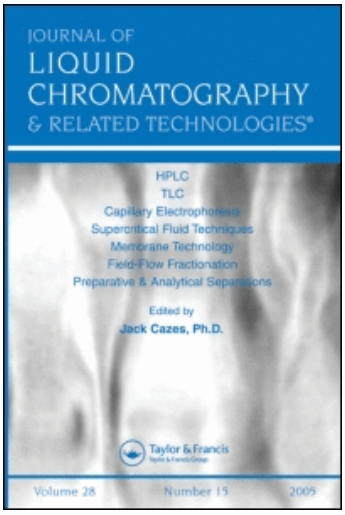


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# Detection of Sulfamethazine Residues in Milk by High Performance Liquid Chromatography

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# DETECTION OF SULFAMETHAZINE RESIDUES IN MILK BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABATRACT

A simple high performance liquid chromatographic (HPLC) procedure for the detection of sulfamethazine residues in milk is described. Milk is extracted with chloroform, the extract evaporated to dryness and then redissolved in potassium phosphate buffer (pH 5.0). The chloroform extract, in buffer, is passed through a cyclobond I solid phase extraction (SPE) column. The SPE column is washed with 10 ml potassium phosphate buffer and then sulfamethazine is eluted with 2 ml aqueous (50%) methanol. The eluent is directly analyzed by HPLC with uv detection at 265 nm. The recoveries ranged from 83.2% to 88.2% in samples fortified between 5 to 40 ppb levels.

## INTRODUCTION

Sulfonamides, including sulfamethazine, are extensively used as veterinary drugs for the treatment of a variety of bacterial infections (1). A recent study by the Food and Drug Administration's National Center for Toxicological Research (NCTR) indicated that sulfamethazine may be a carcinogen (2). As a result, the use of sulfamethazine in lactating cows is no longer permitted.

A number of chemical methods have been reported for the detection of sulfonamide residues in milk (3,4). High Performance Liquid Chromatography, however, is the most widely used technique for this purpose (5-10). The LC method presently used by FDA for the detection of sulfamethazine residues in milk gives an average recovery of 77% in samples fortified between 5 to 20 ppb levels (10). Also a number of extraneous peaks were present in the LC chromatogram (10).

The method described here involves a liquid extraction using chloroform followed by a rapid solid phase extraction cleanup step to determine sulfamethazine residues as low as 5 ppb level in milk.



## METHOD

Reagents and standards:

a) Chemicals.- Sulfamethazine (Sigma Chemical Co., St. Louis, MO). Potassium phosphate buffers (monobasic and dibasic), ammonium acetate, acetic acid, HPLC grade methanol and chloroform (Fisher Chemical Co., Fairlawn, NJ).

b) Potassium phosphate buffer.- 13.60 gm monobasic potassium phosphate was dissolved in 100 ml distilled water and pH adjusted to 5.0 with dibasic potassium phosphate.

c) Mobile phase for LC.- 50 millimolar ammonium acetate buffer was prepared by dissolving 3.85 gm ammonium acetate in 900 ml water and pH adjusted to 4.7 with acetic acid. Final volume was made to 1000 ml with distilled water. LC mobile phase was prepared with 700 ml buffer and 300 ml methanol.

d) Sulfamethazine standard solutions.- Sulfamethazine (100 mg) was dissolved in 100 ml methanol. One ml of this solution was made to 100 ml in a volumetric flask with distilled water (solution B).

e) Working standard.- Ten ml of solution B was diluted to 100 ml with distilled water to provide a solution with a concentration of 1000 ng/ml.

f) Fortification of milk.- Milk samples were fortified with sulfamethazine at 5, 10, 20 and 40 ppb levels by diluting 50, 100, 200 and 400 ul of the working standard to 10 ml with milk.

Apparatus:

a) Solid Phase Extraction Columns.- Cyclobond I, SPE columns 3 ml size (Advanced Separation Technologies Inc., Whippany, NJ).

b) Liquid Chromatograph.- A Constametric III solvent delivery pump equipped with a 7125 Rheodyne injector and a SpectroMonitor III variable wavelength uv detector was used (LDC/Milton Roy, Riviera Beach, FL). A HP 3390 integrator was used for quantification.

c) LC Column.- LC-18-DB, 25 cm X 4.6 mm, 5um particle size (Supelco Inc., Bellefonte, PA).



Procedure:

Milk (10 ml) was pipetted into a 125 ml separatory funnel and 50 ml chloroform added. The mixture was shaken vigorously for one minute and allowed to stand for 2-3 minutes. The shaking was repeated three times and the chloroform extract was filtered through whatman No. 2 filter paper into a 125 ml pear shaped flask. The milk remaining in the separatory funnel was extracted again with 25 ml chloroform in a similar manner and filtered into the same pear shaped flask. The combined chloroform extracts were evaporated to dryness on a rotatory evaporator while maintaining the temperature below 40°C. Five ml of the potassium phosphate buffer (pH 5.0) was added to the flask and the flask shaken

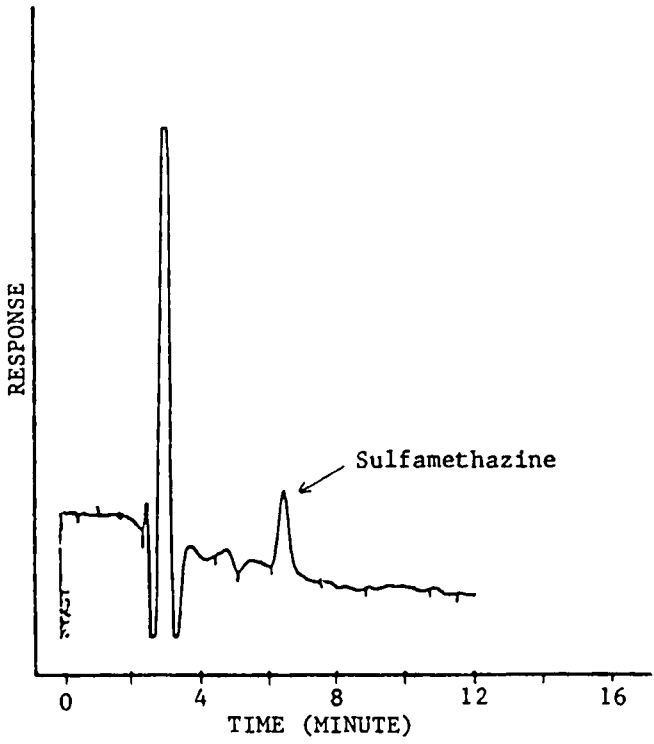


Figure 1. Chromatogram of standard sulfamethazine (1.25 ng).



vigrously on a vortex mixer three to four times at intervals of about 1-2 minutes.

A cyclobond I, SPE column was conditioned by first washing with 5 ml distilled water followed by 5 ml potassium phosphate buffer. The chloroform extract of milk in buffer was passed through the SPE column. The flask was washed with additional 5 ml of buffer and the washings were also passed through the same SPE column. The SPE column was then washed with additional 10 ml buffer. Sulfamethazine, which was retained

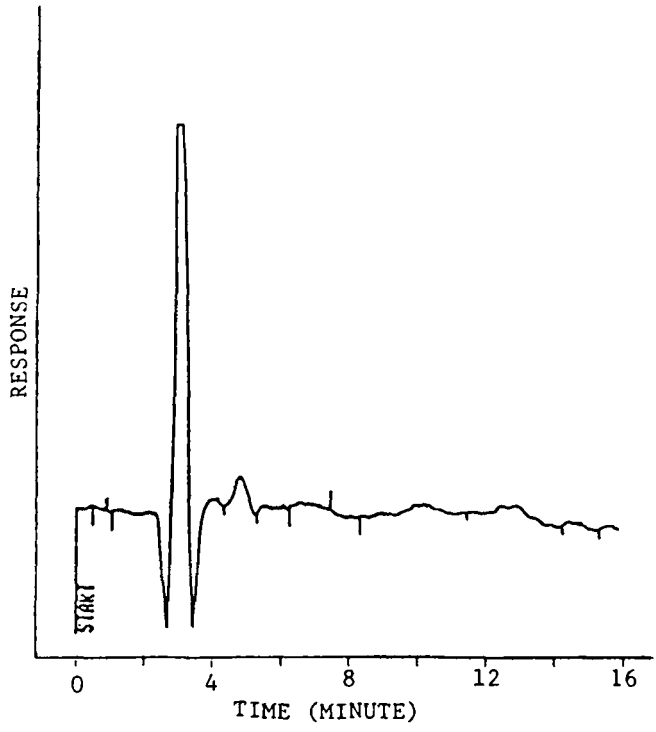


Figure 2. Chromatogram of milk sample without fortification with sulfamethazine.



on the column, was eluted with 2 ml aqueous (50%) methanol. The total eluent was made to 2 ml with aqueous methanol and 25 - 50 ul was injected into the HPLC.

Liquid Chromatography:

The mobile phase flow rate was set at 1.3 ml/minute. The UV detector was set at 265 nm with sensitivity setting at 0.01 AUFS. Quantification was based on peak height measured by HP 3390 integrator.

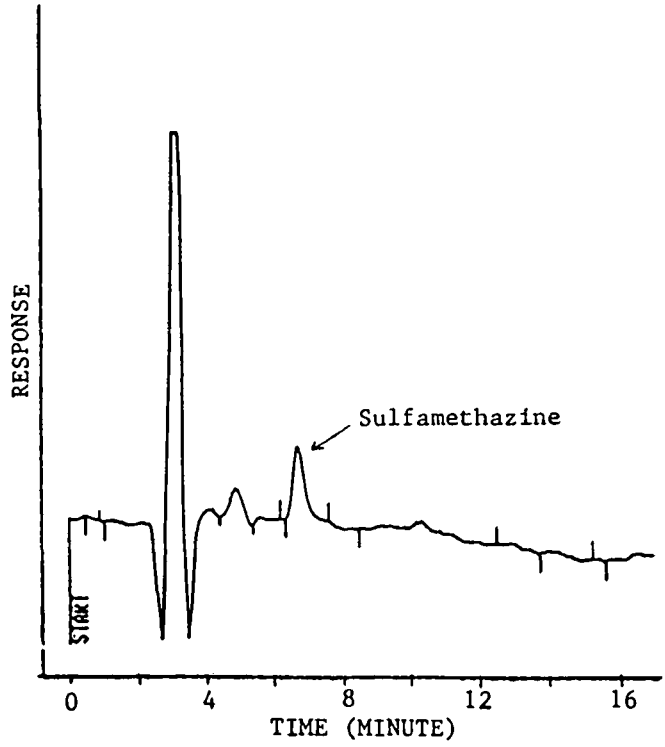


Figure 3. Chromatogram of milk sample fortified with sulfamethazine at 10 ppb level.



## RESULTS AND DISCUSSION

The calibration plot for the standard sulfamethazine was obtained by plotting the mean peak height versus the concentration of sulfamethazine with concentrations ranging from 1 to 50 nanograms. The plot was linear with a correlation coefficient of 0.988.

Figure 1 shows a representative chromatogram of the 1.25 ng sulfamethazine standard with a retention time of 6.5 minutes. An unfortified milk sample was extracted with chloroform, cleaned-up on a

TABLE 1
Recoveries of sulfamethazine added to milk at 5, 10, 20 and 40 ppb levels.

| Fortification levels ppb | Recovery % |
|---|---|
| 5 | 81.6 |
| 5 | 89.2 |
| 5 | 78.8 |
| Average ± SD | 83.2 ± 4.4 |
| 10 | 91.3 |
| 10 | 88.5 |
| 10 | 78.3 |
| Average ± SD | 86.0 ± 5.6 |
| 20 | 88.5 |
| 20 | 82.5 |
| 20 | 93.6 |
| Average ± SD | 88.2 ± 4.5 |
| 40 | 90.5 |
| 40 | 86.2 |
| 40 | 81.0 |
| Average ± SD | 85.9 ± 3.9 |



cyclobond I SPE column as detailed in the procedure, and examined on LC. The LC chromatogram shows no peak eluting at the retention time of sulfamethazine (Figure 2). The milk samples fortified with sulfamethazine at different levels (5 to 40 ppb) were also extracted, cleaned-up and examined on LC. Figure 3 shows a representative chromatogram of milk sample fortified at 10 ppb level with a well resolved sulfamethazine peak at 6.5 minutes.

The recoveries of sulfamethazine from the fortified milk samples, based on the peak heights, are given in Table 1 and ranged from 83.2% to 88.2% (SD 3.9 to 5.6) in samples fortified between 5 to 40 ppb levels.

In the method presently being used by FDA for the analysis of sulfamethazine residues in milk (10), the chloroform extract was evaporated to dryness and the concentrated extract was partitioned between 5 ml hexane and 1 ml potassium diphosphate buffer. Sulfamethazine was thus dissolved in aqueous buffer and examined by HPLC. The LC chromatogram in this method showed a number of extraneous peaks. To eliminate these extraneous peaks, the chloroform extract was cleaned-up by a quick solid phase extraction before HPLC analysis.

The cyclobond I SPE column was chosen because of its ability to retain sulfonamides. Cyclobond I SPE column contains βCyclodextrin bonded to 40 micron silica which can form an inclusion complex with sulfonamides in aqueous solution (11, 12). This inclusion complex between cyclodextrin and sulfonamides is very pH dependent and the maximum stability of the complex is near pH 5.5 (12). Potassium phosphate buffers, with varying pH and ionic strengths, were tested to optimize the maximum retention of sulfamethazine on the SPE column. A one molar potassium phosphate buffer at pH 5.0 was found to be the most efficient for this purpose. Therefore, the chloroform extract of milk was dissolved in this buffer before passing through the SPE column. Sulfamethazine, which was retained on the SPE column, can then be eluted with aqueous (50%) methanol.

In conclusion, the proposed method describes a simple, fast and reliable approach for the detection of sulfamethazine residues in milk up to 5 ppb level. Research efforts are under way to extend this method for the determination of other sulfonamide residues in milk.

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