

DRUGS IN FEEDS

Quantitative Colorimetric Method for Sulfamethazine in Swine Feeds

DANIEL P. SCHWARTZ

U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, Philadelphia, PA 19118

Sulfamethazine (SM) in swine feeds is quantitated colorimetrically by using a relatively simple procedure. The drug is isolated from an extract of feed by trapping it on a small bed of anion-exchange resin buffered at pH 7.9 following the removal of possible interfering compounds by pre-columns. SM is then eluted, diazotized, and coupled using the Bratton-Marshall reagents. The intensity of color developed is determined spectrophotometrically at 540 nm and the concentration of SM is calculated using a molar absorptivity of 52 500. Feeds containing from 1 to 130 ppm can be analyzed with very good accuracy and precision. The method has satisfactory specificity, is inexpensive, and can be applied to a relatively large number of samples per day. A simple and safe method for preparing *N*-methyl SM at the low microgram level is described together with a thin layer chromatographic system for tentatively verifying the presence of SM in the feed both as the underivatized drug and as *N*-methyl SM.

Sulfamethazine (SM) is added to swine feeds to help in disease prevention. Despite the widespread use of this drug and the concomitant incidence of tissue violations of the regulatory threshold, relatively few quantitative methods have been proposed for SM in feeds (1-6). The trend in methodology has been heavily toward chromatographic quantitation in the belief that this will result in greater specificity.

In a previous report from this laboratory (7), we described a rapid colorimetric screening method for SM in swine feeds. The method specified a small bed of anion-exchange resin buffered at pH 7.9 to trap the SM after removal of possible interfering components. SM was then eluted and detected using the Bratton-Marshall reagents. We now report a modification of that procedure which is applicable to the quantitative determination of SM in swine feeds in a practical, convenient, and specific manner and over a wide range of SM concentrations.

METHOD

Reagents

Deionized or distilled water was used throughout the study. All solutions were aqueous.

(a) *Sodium nitrite*.—0.12%.

(b) *Ammonium sulfamate*.—0.8%.

(c) *N-1(Naphthyl)ethylenediamine dihydrochloride (NED)*.—(Sigma Chemical Co., St. Louis, MO 63178) 0.8% containing 0.1% ethylenediaminetetraacetic acid (EDTA).

Solutions (a), (b), and (c) were conveniently stored in, and dispensed from, drop dispenser bottles (NALGE 2411 Series, A. H. Thomas Co., Philadelphia, PA 19105). Solutions were kept at 4°C when not in use and were usable for 2 months.

(d) *Potassium dihydrogen phosphate*.—0.2M. 27.8 g $\text{KH}_2\text{PO}_4/\text{L}$.

(e) *Dibasic sodium phosphate*.—0.2M. 71.1 g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}/\text{L}$.

(f) *pH 7.9 buffer*.—Using pH meter, add (d) to 200 mL of (e) until pH 7.9-7.95 is obtained.

(g) *pH 5.7 buffer*.—Using pH meter, add (e) to 200 mL of (d) until pH 5.65-5.75 is obtained.

(h) *Acidic alumina*.—(Fisher Scientific Co., Cat. No. A-948). Keep tightly closed when not in use. Do not substitute.

(i) *Resin*.—AG MP-1 100-200 mesh, chloride form (Bio-Rad Laboratories, Richmond, CA). Do not substitute.

(j) *Sea sand*.—(Fisher, Cat. No. S-25).

(k) *Triton X-100*.—(Sigma).

Apparatus

(a) *Transfer pipets*.—Polyethylene "jumbo bulb" pipets (Fisher, Cat. No. 13-711-7). Cut off ca 1 cm from top of bulb portion.

(b) *Pipet tips*.—5 mL (Rainin Instrument Co., Woburn, MA 01801).

(c) *Centrifuge tubes*.—50 mL polypropylene, disposable (Fisher, Cat. No. 05-538-60).

(d) *Glass beads*.—Solid 3 mm and 4 mm (A. H. Thomas Co., Cat. Nos. 5663-L13 and 5663-L19, respectively).

(e) *Melting point capillaries*.—(Thomas, Cat. No. 6418-F10).

Optional Reagents and Apparatus

Dissolve 1 g Diazald (*N*-methyl-*N*-nitroso-*p*-toluenesulfonamide, Aldrich Chemical Co., Milwaukee, WI) in 5 mL 2-(2-ethoxyethoxy)ethanol (stable for at least 6 months in freezer); 50% aqueous NaOH; Chromosorb 102, 100-120 mesh (Sigma); silica gel G plates, 250 μm , 2.5 \times 10 cm (Analtech, Inc., Newark, DE); shell vials, ¼ dram (VWR Scientific, PO Box 8188, Philadelphia, PA); sleeve-type septa, 6.7 \times 12 mm (Fisher, Bittner No. 713120).

Procedure

Preparation of pH 7.9 and pH 5.7 resins.—Magnetically stir 20 g AG-MP-1 resin for 1 h with \geq 200 mL pH 7.9 phosphate buffer. Filter suspension on 60 mL coarse sintered glass funnel or through Whatman No. 3 paper on Buchner funnel, and remove excess fluid by vacuum suction. Wash resin with ca 200 mL water, remove excess water by suction, and transfer resin to 250 mL screw-cap bottle with 50 mL water and 50 mL 95% ethanol. Prepare pH 5.7 resin in similar fashion as pH 7.9 resin, except use pH 5.7 buffer. Both preparations are usable for at least 2 months when bottles are stored at 4°C, with screw caps tightened.

Extraction of feed.—Feed sample must be homogeneous and fine. (We use an inexpensive coffee mill (Waring Products, Route 44, New Hartford, CT 06057).) Grind coarse and nonhomogeneous samples 1 min. Accurately weigh 5.0 g \pm 10 mg sample of ground feed, transfer into 50 mL polypropylene centrifuge tube, and cover with 25 mL (pipet) acetone-methanol-water (85 + 10 + 5) (hereafter called solvent). Shake tube a few times to wet all particles, then store \geq 3 h or, more conveniently, overnight (16-18 h) in the dark. Then shake tube a few times, and let stand 10-15 min for particles to resettle.

Preparation of pH 7.9 tube.—Loosely place small plug of fine glass wool about halfway into tapered section of cut-off

Received May 4, 1984. Accepted July 18, 1984.

Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

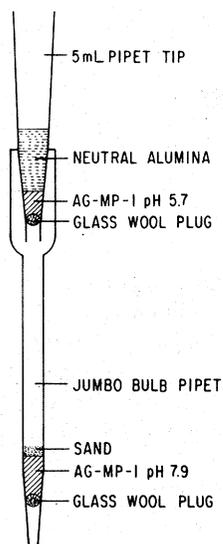


Figure 1. Setup for quantitative isolation and purification of sulfamethazine from feed extract.

transfer pipet. Shake and then swirl pH 7.9 resin, and while it is in suspension, pipet 1.5 mL into tube, and let tube drain. Wash excess resin from sides with water, let drain, and add about 300 mg sand. Exact same amount of resin is probably not obtained each time in the 1.5 mL aliquot, using hand swirling to affect suspension of resin. However, slight variation in amount of resin taken apparently does not affect quantitative recovery of amounts of SM investigated in this study. Use of a magnetically stirred suspension of resin at this point is not recommended because stirring bar will gradually fragment the resin beads, and this will eventually cause turbidity in the solution to be read spectrophotometrically. Other mechanical methods of obtaining the resin suspension, such as shaking or vortex-mixing, may be helpful but have not been investigated.

Preparation of pH 5.7 alumina tube.—Plug 5 mL pipet tip with small wad of glass wool near bottom of tapered portion. Pipet 1.5 mL of pH 5.7 resin suspension taken as described above, let drain completely, and add 1.45–1.50 g alumina. Place pipet tip piggyback in pH 7.9 tube (Figure 1).

Isolation of SM from feed extract.—Pipet 5 mL aliquot of feed extract down side of upper tube. Let first ca 0.5 mL run slowly into tube so that displaced air can escape, then add remainder at normal pipet speed. (If cracks appear in the alumina bed which stop or greatly reduce percolation, gently stir to remove air bubbles by using closed end of melting point capillary, then leave capillary in tube.) After all of solution has drained through both tubes, wash sides of upper tube with 1 mL solvent, and when this has drained, pipet 2 mL solvent into upper tube and let drain. Remove upper tube, pipet 2 mL solvent to wash sides of lower tube, and let drain. Squirt water down side of tube (do not disturb bed) until level reaches beginning of bulb portion and let drain.

Determination of SM

Place pH 7.9 tube in neck of ≥ 5 mL volumetric flask and wedge a melting point capillary between tube and neck to let air escape. Carefully pipet 4 mL 3.5N HCl containing 0.05% Triton X-100 slowly down side to elute SM. Add 2 drops of sodium nitrite solution to flask, mix by shaking, and let stand 2 min. Add 2 drops of ammonium sulfamate solution, mix, and let stand 1 min. Finally, add 2 drops of NED solution, mix, and let stand ≥ 15 min, but < 1 h in the dark. Dilute to

mark with water and read at 540 nm against a reagent blank. If absorption is > 0.4 AU, dilute with water. Calculate concentration of SM in feed using the formula:

$$\text{ppm SM} = (A_{5 \text{ mL}} \times 278.3 \times \text{DF})/10.5$$

where A = absorbance at 540 nm, 278.3 = MW of SM, DF = dilution factor, and 10.5 = absorbance of 1 μM SM in 5 mL, based on a molar absorptivity of 52 500.

This equation can be simplified to:

$$\text{ppm SM} = A_{5 \text{ mL}} \times 26.5 \times \text{DF}$$

Confirmation by Thin Layer Chromatography (Optional)

If desired, SM on pH 7.9 resin can be isolated and subjected to thin layer chromatography (TLC). Elute the resin with 4 mL 3.5N HCl (no Triton X-100). Add 0.9 mL concentrated NH_4OH to the effluent, mix, and let cool. Add 2 mL saturated solution of KH_2PO_4 . Mix with melting point capillary and touch the end to pH indicating paper. If pH is not between pH 4 and pH 6, add 1 additional mL KH_2PO_4 solution. Prepare small column of Chromosorb 102 as follows: Drop 3 mm glass bead into transfer pipet. Add 200–250 mg sand and about 300 mg Chromosorb 102. Wash column with 4 mL acetone, add 400–500 mg sand, then wash with two 4 mL portions of water. Apply neutralized 3.5N HCl effluent solution, and when drained, wash down sides with ca 1 mL water. Then fill transfer pipet with water, let drain, and force excess water out by pressure or vacuum application. Elute column with 1.4 mL acetone and collect effluent in shell vial. Evaporate acetone using stream of nitrogen, add 25 μL acetone to residue, and apply part or all to origin of TLC plate along with authentic SM. Develop plate in methanol until solvent is about 1 cm beyond origin. Remove, air dry until methanol is visibly evaporated, and redevelop plate until solvent (ethyl acetate–chloroform–methanol, 2.5 + 2.5 + 0.5) has moved about halfway up plate (ca 4 min). Detect spots after drying by exposing plate to nitrogen oxide vapors generated when solid NaNO_2 is added to dilute H_3PO_4 , and then by spraying with 0.3% NED in methanol (8).

Preparation of *N*¹-Methyl Sulfamethazine

A simple and safe procedure for making *N*¹-methyl derivatives of SM and other sulfa drugs is based on the capillary procedure described by Schwartz and Bright for making methyl esters of organic acids (9). A melting point capillary open at both ends (1.5–1.8 \times 100 mm) is pushed into a compact wad of glass wool (Thomas, Cat. No. 5745-C10) contained in a vial. The capillary is twisted until a small plug is retained. The plug is pushed into the capillary with a wire to a distance of ca 4 cm. Then it is dabbed into Chromosorb 102 until some of the powder is retained. The capillary is inverted and tapped on a solid surface to settle the powder against the glass wool plug. The process is repeated with the Chromosorb until a column of powder ca 3 cm is made. The powder is pressed into a compact column by tamping with wires, pushing from both ends of the bed. The acetone solution of the residue obtained from the TLC step is applied to the glass wool-free end of the bed in ca 5 μL aliquots so that a total of 1 to 5 μg of the sulfa drug is deposited. All of the acetone solution can be applied rapidly by pulling a slight vacuum on the glass wool end of the capillary while holding the thumb and index finger on the wetted portion of the capillary. This volatilizes the acetone almost instantly and another aliquot is deposited, etc., and only a portion of the bed has actually been wetted. The glass wool-free end is then pushed into the vial of glass wool, while holding the vial above the capillary, and the plug obtained is pushed against the bed. After this operation there

Table 1. Analysis of swine feeds fortified with sulfamethazine (SM)

Feed	Initial SM concn, ppm	Added SM, ppm	Other drugs added (ppm)	Recovery, % \pm SD (n)
A	\sim 0.5	130		97.3 \pm 2.6 (6)
B	<0.2	3.5		96.4 \pm 3.4 (4)
		1.9		102.9 \pm 3.5 (4)
		5.3		97.1 \pm 1.3 (4)
		105.9		99.4 \pm 2.6 (6)
C	<0.2	0.7		120 \pm 8.3 (6)
		2.6		
		1.3	trimethoprim (180) procaine (280) trimethoprim (90)	99.8 \pm 1.3 (4)
D	<0.2	1.6	procaine (140)	101.5 \pm 3.8 (4)
			carbadox (50)	99.0 \pm 3.5 (6)
E	<0.2	66.7	terracycline (600)	98.7 \pm 0.9 (4)
F	\sim 0.3	1.0		103 \pm 4.0 (4)
G	0	51.1		95.8 \pm 0.8 (8)
		0.7		108.6 \pm 1.1 (4)
H	\sim 0.6	7.1		101.9 \pm 4.4 (4)
I	<0.2	5.0	chlortetracycline (450)	100.4 \pm 1.9 (6)
J	<0.2	0.9		95.0 \pm 2.3 (6)
K	\sim 0.3	9.7	oxytetracycline (1200)	97.9 \pm 1.6 (6)
L	<0.2	22.0		95.9 \pm 0.4 (4)
M	<0.2	3.1	lincomycin (1600)	103.0 \pm 2.3 (6)

should be ca 1.0–1.5 cm space between the plug and end of the capillary. A stirring bar is made by cutting ca 0.5 cm wire from a paper clip, and the bar is placed in a 2 mL vial along with 1 drop of 50% NaOH. The sleeve portion of a rubber septum, previously pierced with an 18 gauge hypodermic needle, is pulled over the vial mouth, and the capillary is inserted (wetted end down), so that the end of the capillary is about halfway into the vial. Diazald solution (ca 50 μ L) is injected through the side of the septum, and the contents are stirred magnetically 10 min. The capillary is withdrawn, aerated ca 1 min in the hood, and ca 30–50 μ L acetone is injected into it. Slight air pressure can be used to force the solvent over the bed, if desired. A convenient apparatus for this is a rubber bulb (Thomas Cat. No. 1957-K10) fitted with a septum, or better, a capillary holder such as those supplied with Microcaps. The first 7–8 μ L (ca 7–8 mm) of effluent that emerges contains all of the derivative. It can then be either spotted directly at the origin of a TLC plate, and developed as described above along with authentic *N*¹-methyl sulfa drug(s), or it can be taken up in a 10 μ L syringe, and injected into a gas chromatograph or GC-MS system using conditions described in the literature (10, 11).

Results and Discussion

Feeds

We attempted to obtain a variety of swine feeds for the study. Because anion-exchange is the basis for the isolation and purification of SM, we considered the possibility, however remote, that an anion or potential anion could occur in feeds in relatively high concentration, which might coextract, escape the trap columns, and displace SM from the pH 7.9 resin. This did not occur with any of the 13 feeds examined. Although the composition of most of the feeds was unknown to us, most were visibly different both in color and texture before grinding. Three of the feeds were commercial samples with labeled analysis. Only one feed was found that was completely devoid of SM before spiking. All of the other feeds contained SM which was verified by cochromatography with authentic SM, and, when treated with diazomethane, cochromatography with *N*¹-methyl SM. This included a feed sample produced in a plant in which SM was claimed to be never on the premises. The feeds examined are designated by letter in Table 1.

Extraction

While studying the effect of grinding feeds on the recovery of SM, we observed that the finer the particles produced during grinding, the longer it took to extract the SM quantitatively when the ground feed was mechanically shaken with the solvent. In view of this observation, the recommended extraction procedure was thoroughly investigated; this revealed that the SM in all feeds, regardless of their degree of fineness, could be quantitatively extracted merely by being wetted by the solvent and left \geq 3 h. We usually left the samples overnight (16–18) in the dark. This method of extraction eliminates the need for mechanical shaking, which was found to have no advantage over the static method.

Additives

The effect of a number of additives in feeds containing SM on the recovery of SM was also studied. These included compounds that do not give a color in the Bratton-Marshall reaction. They were included to determine whether they could reduce recovery of SM by (a) interfering in the extraction, (b) reacting with SM in the course of its isolation, or (c) interfering with the exchange of SM on the pH 7.9 resin. The additives are indicated in Table 1. The feeds were fortified with the additives in the same way SM was added (see below). There was no observed interference from any additive at the concentrations studied. A number of the additives may normally be present in the feed along with SM.

Fortification of Feeds with SM

The mode of addition of SM to the feed for recovery studies was examined. When accurately weighed samples of SM (99.5% Cyanamid, previously dried to constant weight) were incorporated into relatively large amounts of feed, quantitative recovery was obtained. However, when the amount of feed available for spiking was limited and a relatively low spike level was desired, this approach was considered to be potentially erroneous because of the very small amounts of SM that had to be accurately weighed and transferred. To circumvent this situation, relatively large (about 100 mg) amounts of accurately weighed SM were ground onto accurately weighed Celite 545 (Fisher), and these were diluted with Celite 545 for addition to the feed so that the ratio of feed to Celite was at least 100:1. All grinding operations were done with mortar and pestle for 5–10 min. We found no

significant differences in recoveries of SM from feed fortified directly with SM, or indirectly via the Celite carrier.

Recovery Data

The data in Table 1 indicate that the method gives satisfactory accuracy and very good precision in all analyses of feeds containing from 1 to 130 ppm SM. Below 1 ppm, recoveries were sometimes erratic, and both the accuracy and precision were less than satisfactory, especially in feeds containing relatively high initial SM values. Because it may be difficult to obtain SM-free feed from a farm normally feeding SM-medicated feed, a value of < 1 ppm may not be a true reflection of the SM content. It is also recommended that if a value of > 130 ppm is obtained for a feed, then an aliquot of the feed extract should be diluted accordingly with extraction solvent so that a 5 mL aliquot contains \leq 130 ppm before analysis. We have analyzed one sample of feed being fed to swine, which contained > 400 ppm SM.

Specificity

A number of compounds capable of giving a positive response in the Bratton-Marshall reaction were studied as possible interferences. These included 95 ppm sulfathiazole, 10 ppm *p*-aminobenzoic acid, 10 ppm anthranilic acid, 5 ppm sulfanilic acid, 8 ppm sulfanilamide, and 5 ppm kynurenine. These were added individually to the feed extract with and without added SM in it. None of the compounds tested escaped the trap layers in the upper tube and did not interfere. Sulfadoxine and sulfadimethoxine used in poultry feeds will interfere, but these drugs are not used for swine. Thus, under normal swine feeding conditions, our procedure has very good specificity for SM.

Effect of Light

We observed that some ground feeds in transparent polyethylene bags gave diminished recovery of SM when the bags were exposed to sunlight either directly or through window glass. The magnitude of the loss of SM varied considerably among the feed samples in which this phenomenon was observed. One sample lost over 50% of its SM in 3 days; another lost only 12% in 5 weeks. We did not attempt to study the effect of light in detail, but precautions were taken to keep SM-fortified feed samples in the dark and cold when not in use. From a practical standpoint, the possible loss of SM

in some feeds because of light could be important to swine producers. It also raises the question of the reaction mechanisms leading to loss of SM in light-susceptible feeds and to the nature, fate, and possible toxicity of the photo-induced products.

Conclusions

The major advantages of the method are simplicity, economy, and speed. Twelve analyses (e.g., 3 samples weighed in duplicate 2 aliquots/sample) can be completed in 2 h by one analyst. When properly set up, only a few square feet of laboratory space is required. We use a test tube rack to hold the tandem column system. The rack is positioned on a suitable receptacle, such as a pail or chromatography jar, which collects the effluent for disposal. The method also uses optionally disposable materials. We estimate the cost of materials to be \$0.50 per analysis with disposal of all plasticware.

The method has the potential to be conducted in the field using a portable colorimeter, or, with lower accuracy, using the permanent color standards described earlier (7).

Acknowledgments

The author thanks B. Schwab, FSIS, Beltsville, MD, and R. Munns, FDA, Denver, CO, for supplying some of the feed samples, and W. Fiddler of this laboratory for suggesting the problem.

REFERENCES

- (1) Horwitz, W. (1981) *J. Assoc. Off. Anal. Chem.* **64**, 104-129
- (2) Allred, M. C., & Dunmire, D. L. (1978) *J. Chromatogr. Sci.* **16**, 533-537
- (3) Munns, R. S., & Roybal, J. E. (1982) *J. Assoc. Off. Anal. Chem.* **65**, 1048-1053
- (4) Cieri, U. R. (1980) *J. Assoc. Off. Anal. Chem.* **63**, 727-735
- (5) Stringham, R. W., Mundell, E. C., & Smallidge, R. L. (1982) *J. Assoc. Off. Anal. Chem.* **65**, 823-827
- (6) Holder, C. L., Thompson, H. C., Jr, & Bowman, M. C. (1981) *J. Chromatogr. Sci.* **19**, 625-632
- (7) Schwartz, D. P. (1982) *J. Assoc. Off. Anal. Chem.* **65**, 701-705
- (8) Parks, O. W. (1982) *J. Assoc. Off. Anal. Chem.* **65**, 632-634
- (9) Schwartz, D. P., & Bright, R. S. (1974) *Anal. Biochem.* **61**, 271-274
- (10) Manuel, A. J., & Steller, W. A. (1981) *J. Assoc. Off. Anal. Chem.* **64**, 794-799
- (11) Suhre, F. B., Simpson, R. M., & Shafer, J. W. (1981) *J. Agric. Food Chem.* **29**, 729-732