5.1.58

AOAC Official Method 999.16
Sulfamethazine in Animal Feeds
Liquid Chromatographic–Post-Column Derivatization Method
First Action 1999

(Applicable for analysis of sulfamethazine in medicated swine and cattle feeds in the range of 0.005–0.22%. Method is not intended for analysis of sulfathiazole.)

Caution: *See* Appendix B, "Laboratory Safety." The following materials used in the method should be considered toxic and care should be taken to avoid skin contact and inhalation of vapors or dust: *p*-dimethylaminobenzaldehyde (causes eye and skin irritation, reacts with tissue amines to turn skin yellow), and hydroquinone (carcinogen, air and light sensitive).

See Table **999.16A** for results of the interlaboratory study supporting the acceptance of the method.

A. Principle

Ground feed, with sulfamerazine added as internal standard, is extracted with 0.2M HCl and 1.5% diethylamine in 25% methanol. Sulfamethazine is separated by LC and measured at 450 nm using post-column derivatization with dimethylaminobenzaldehyde.

B. Apparatus

(**a**) *Liquid chromatograph*.—Instrument maintaining constant pulseless flow of mobile phase with optional autosampler. Injection volume, 10–200 µL (*see* **D**, System Suitability Tests); mobile phase flow rate, 1–1.3 mL/min.

(**b**) *Detector*.—Wavelength, 450 nm. Sensitivity to meet system performance characteristics in **D**, System Suitability Tests. Adjustable rise-time and autozero feature are desirable. Shimadzu SPD-6AV UV/Vis detector is suitable, (Shimadzu Corp., 7100 Riverwood Dr, Columbia, MD 21046, USA).

(**c**) *Chromatographic column*.—Any reversed-phase column, C_{18} or C_8, that meets performance characteristics in **D**, System Suitability Tests. Use a guard column. Phase Separation's Spherisorb ODS2 (5 µm, 250 × 4.6 mm id); GL Science's Inertsil ODS2, 5 µm, 25 cm; and Macherey-Nagel's Nucleosil 120A, 5 µm, 25 cm are suitable columns.

(**d**) *Post-column reagent pump*.—Any pump maintaining a constant, low pulse flow of reagent from 0.5–1.5 mL/min. Teflon-based pump seals may be necessary. Pressure maximum >2000 psi (14 MPa) desirable. The following PCD systems are satisfactory: Eldex A-30-S metering pump with Fluoroloy K seals (Fisher or equivalent); Pickering Laboratories Flow Reagent Conditioner; Scientific Systems, Inc. Model LP-21 Lo-Pulse pulse damper between the reagent pump and the Pickering device (Fisher or equivalent).

(**e**) *Mixing coil*.—Teflon, 1.5 mm (1/16 in.) od, 0.5 mm id × 10 ft.

Prepare Teflon tubing for connecting to normal 1/16 in. (1.5 mm) stainless steel fittings as follows: Place 1/16 in. (1.5 mm) nut and ferrule on each end of tubing. Buff sharp edges off diagonal tip of 1 in. No. 23 disposable syringe needle, and insert needle into each end of tubing up to Luer fitting (avoid digging needle into tubing). Twist Luer fitting back and forth to break fitting from needle (make sure needle remains open). Push needle in flush with end of Teflon tubing by pressing broken end of needle against metal surface. Slide ferrule and nut over inserted needle and attach between mixing tee and detector. Coil configuration does not affect chromatography. (*Note*: It is not always necessary to insert needle; sometimes a direct connection of the Teflon tubing to the stainless steel fitting is possible.)

(**f**) *Mixing tee*.—A 1/16 in. (1.5 mm), zero dead volume device for combining the column and post-column reagent effluent into a single stream for delivery into mixing coil. Attach column and reagent effluent lines opposite from each other for best mixing. A Valco 1/16 in. (1.5 mm) ZDV Tee (P/N ZT1C), 0.25 mm id is suitable.

(**g**) *Clarification filter*.—Insert 13 mm glass fiber pre-filter pads (Gelman, or equivalent) into 0.5 in. id syringe barrel (e.g., 6cc "Monoject," Sherwood Medical, 1915 Olive St, St. Louis, MO 63103, USA) or any other filtration device designed for clarifi-

Table 999.16A Interlaboratory study results for determination of sulfamethazine in swine and cattle feed by liquid chromatography with post-column derivatization

Feed	No. labs	SMT[a], %	s_r[b]	r[c]	RSD_r,[d] %	s_R[e]	R[f]	RSD_R,[g] %
Swine 1	10 [1][h]	0.22 (0.19)	0.00052	0.0015	0.28	0.0023	0.0065	1.3
Swine 2	10	0.11 (0.11)	0.0036	0.0099	3.1	0.0036	0.010	3.2
Cattle 1	10	0.0154 (0.014)	0.00043	0.0012	3.1	0.00044	0.0012	3.1
Swine 3	10	0.011 (0.010)	0.00049	0.0014	4.7	0.00049	0.0014	4.7
Swine 4	10	0.011 (0.010)	0.00015	0.00041	1.4	0.00037	0.0010	3.6
Cattle 2	10 [1][h]	0.0077 (0.0068)	0.00014	0.00039	2.0	0.00032	0.00093	4.9

[a] Normal sulfamethazine label claim (mean value from study).

[b] s_r = repeatability standard deviation.

[c] r = repeatability value ($2.8 \times s_r$).

[d] RSD_r = repeatability relative standard deviation.

[e] s_R = reproducibility standard deviation.

[f] R = reproducibility value ($2.8 \times s_R$).

[g] RSD_R = reproducibility relative standard deviation.

[h] Number of outliers in brackets.

cation of aqueous chromatographic solutions, e.g., Nylon 66 syringe tip filters (0.2 μm).

(**h**) *Centrifuge*.—Device to centrifuge 50 mL tubes at 1200 × *g*.

C. Reagents

(**a**) *Water*.—LC grade.

(**b**) *Methanol*.—LC grade.

(**c**) *Acetic acid*.—2%. Dilute 20 mL glacial acetic acid to 1 L with water.

(**d**) *Acetonitrile*.—LC grade.

(**e**) *Oxalic acid*.—0.2M. Dissolve 25.2 g oxalic acid dihydrate (FW = 126.07 g) in distilled, deionized water to make 1 L.

(**f**) *Diethylamine*.—1.5%.

(**g**) *Sulfamethazine (SMT) standard*.—USP Reference Standard.

(**h**) *Dimethylaminobenzaldehyde (DMAB)*.—Sigma D 2004, (Sigma Chemical Co., St. Louis, MO) or equivalent.

(**i**) *Hydroquinone (HQ)*.—Aldrich 24,012-5, (Aldrich Chemical Co., Milwaukee, WI), or equivalent.

(**j**) *Sulfamerazine (SMR)*.—Sigma S-8876, or equivalent.

(**k**) *Extractant*.—0.2M HCl + 1.5% DEA, (**f**), in 25% methanol. To 1 L volumetric flask containing 250 mL methanol, (**b**), add ca 300 mL water plus 16.7 mL HCl, and mix. Add 15 mL DEA, dilute nearly to volume with water and mix, adjust to ambient temperature, dilute to volume, and mix. The pH of this extractant is designed for use with a 5 g test portion; the buffering capacity of a larger test portion would require a proportionally larger volume of extractant or higher HCl concentration. The pH of an extract should be close to 2.

(**l**) *Diluent*.—0.15M HCl + 1.5% DEA, (**f**), in 25% methanol. Prepare as in (**k**) except use 12.5 mL HCl.

(**m**) *Internal standard solutions*.—(*1*) *SMR stock solution*.—1 mg/mL. Weigh ca 0.1 g SMR, (**j**), into 100 mL volumetric flask. Dissolve and dilute to volume with extractant. An extra 2–5 drops Hcl and sonication aids dissolution. Solution is stable stored in dark. (*2*) *Internal standard (IS) spiking solutions*.—(*i*) *IS-A: About 100 μg/mL*.—Dilute 10.0 mL SMR stock solution, (**j**)(*1*), to 100 mL with extractant. (*ii*) *IS-B: About 200 μg/mL*.—Dilute 20 mL stock solution to 100 mL with extractant. (*iii*) *IS-C: About 400 μg/mL*.—Dilute 20 mL stock solution to 50 mL with extractant.

(*Note*: Internal standard spikes B and C are only used for feeds with > 0.016% SMT. *See* Table **999.16B**)

(**n**) *SMT standard solutions*.—(*1*) *SMT stock solution*.—About 1.1 mg/mL. Accurately weigh 0.105–0.115 g SMT, (**g**), standard (actual weight = W_s) into 100 mL volumetric flask . Dissolve and dilute to volume with extractant (sonication aids dissolution). Solution is stable stored in dark. (*2*) *SMT intermediate stock solution (SMT ISS)*.—About 55 μg/mL. Dilute SMT stock solution 10.0 mL to 200 mL with diluent.

(**o**) *SMT–SMR working standard*.—About 5.5 μg/mL SMT, (**g**), and ca 5.0 μg/mL SMR, (**j**). Dilute 10.0 mL SMT intermediate stock solution, (**n**)(*2*), plus 5.00 mL SMR IS spiking solution (IS-A), (**m**)(*1*)(*i*), to 100 mL with diluent and mix. Total standard dilution = D_s = 20 000 mL (*see* Table **999.16B**). (*Note*: Solution should be prepared fresh daily. Protect from light when not using.) If extractant rather than diluent is used for dilution, solution will be too acidic and peak shape will deteriorate.

(**p**) *Post-column reagent*.—15 mg/mL DMAB and 0.5 mg/mL HQ. Dissolve 3.0 g DMAB, (**h**), and 0.1 g HQ, (**i**), in 100 mL glacial acetic acid. Carefully add 60 mL methanol, (**b**), and mix well. Add 40 mL water, (**a**), mix well, and filter under vacuum. (Multiples of the given quantities are used if more than a few test samples are to be assayed.) Degas 2–3 min under vacuum while stirring. If stored in dark, solution is stable >1 month. (*Caution*: Avoid contact of reagent with skin or clothing; treat as strong acid.)

Table 999.16B Calibration solutions for sulfamethazine/sulfamerazine

Concn. SMT, μg/mL[a]	SMT ISS[b], mL	IS spike A, mL	Dilute to, mL	D_s[c]
1.1	2.0	5.00	100.0	100000
2.2	4.0	5.00	100.0	50000
5.5	10.0	5.00	100.0	20000
6.6	12.0	5.00	100.0	16667

[a] Actual μg/mL SMT = $\frac{\quad \times \quad}{\quad}$.

[b] SMT intermediate stock solution (ca 55 μg/mL), (**n**)(*2*).

[c] D_s= Total dilution of standard in mL. *See* "Dilution Calculation" in **G**, *Calculations*, for example.

(**q**) *Mobile phase*.—Acetonitrile, (**d**),–2% acetic acid, (**c**), (17 + 83), or as adjusted to optimize chromatography. *Alternative mobile phase*.—Acetonitrile, (**d**),–methanol, (**b**),–2% acetic acid, (**c**), (4 + 16 + 80) if premixed, or [A] acetonitrile, (**d**),–methanol, (**b**), (2 + 3), plus [B] 2% acetic acid, (**c**), with A + B = 20 + 80 if instrument-mixed. Other proportions of acetonitrile and methanol in the organic modifier may be optimum for certain columns. Use of the alternative mobile phase may cause appreciable shifts in the capacity factors of interfering peaks, and thus improve their resolution relative to the sulfonamide peaks. Resolution of sulfonamides on some columns may also be improved by use of the alternative mobile phase.

D. System Suitability Tests

If determined manually, dimensions used for resolution and peak skew calculations should be measured from a chromatogram recorded at fast speed (ca 5 cm/min or 2 in./min) and sensitivity adjusted to give peaks ≥80% full scale.

(*1*) *System resolution*.—Prepare a system resolution standard like the working standard except include sulfathiazole (STZ, Sigma S9876) at about the same concentration as SMR. This is a qualitative test solution and can be used for several months if stored in the dark. Include near beginning and end of each analytical set to verify proper resolution of sulfonamides. Elution order is STZ, SMR, SMT (*see* Figure **999.16**). System (LC, column, and detector) should allow for separation of STZ, SMR, and SMT peaks from each other and from associated co-extracted materials. SMR–SMT peak pair must have baseline resolution, and peaks should not have excessive tailing. STZ ($k' \geq 1$) must be separated well enough from the SMR to be measurable if present in an SMT sample as a contaminant.

(*2*) *System injection precision*.—Make ≥5 replicate injections of working standard (or other solution containing an appropriate concentration of SMT and SMR). The relative standard deviation of SMT/SMR peak response ratio should be <2%.

(*3*) *System linearity*.—Prepare 4-point standard curve over range of SMT concentration from ca 1 to 6 μg/mL (*see* Table **999.16B**). Use same internal standard concentration in each. Plot ratio of analyte to internal standard peak response versus analyte concentration. Plot should be linear and pass through origin. (If confidence

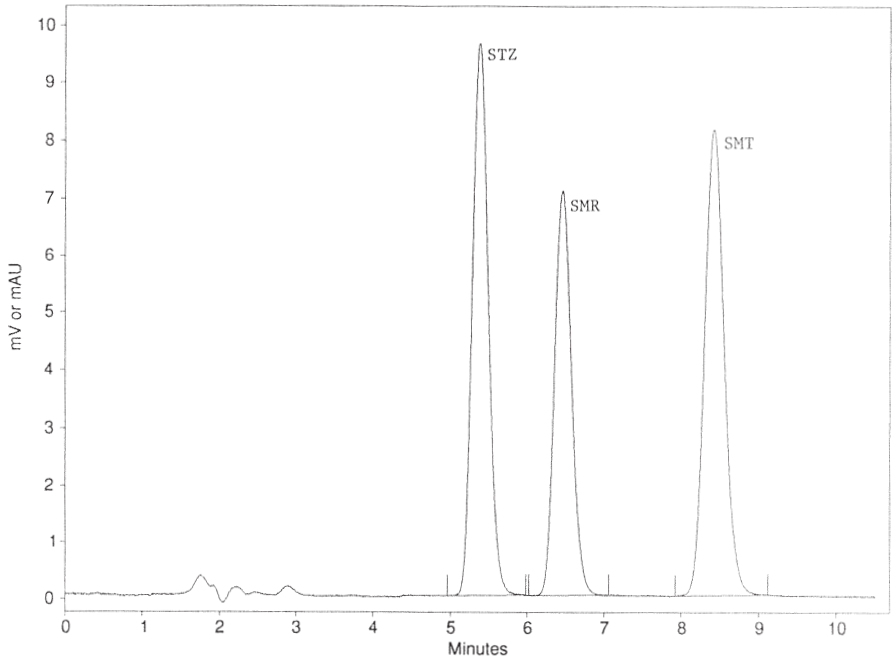

Figure 999.16—System resolution standard chromatogram. Sulfathiazole (STZ), sulfamerazine (SMR) internal standard, and sulfamethazine (SMT). Conditions as in *System Suitability* section.

limits of the intercept include zero, the null hypothesis that the intercept equals zero can not be rejected, i.e., hypothesis is accepted that the line passes through zero.)

(*4*) *System sensitivity*.—When detector sensitivity is set to produce working standard peak height for SMT of 50–80% full scale (if strip chart recorder), height of SMT peak from a 1/10 dilution of working standard should be ≥4 times baseline noise.

E. Extraction

(*1*) Accurately weigh 4.75–5.25 g well-mixed ground test sample, and transfer to 250 mL Erlenmeyer flask. W_u = actual weight of test portion to nearest 0.001 g. SMT-containing feeds are generally made from stabilized premixes and require special grinding considerations to obtain satisfactory analytical repeatability. Grinding to pass a 0.75 mm screen followed by a second grind to pass a 0.5 mm screen in a Brinkmann analytical mill is satisfactory for a 5 g test portion. Test grinding and mixing procedure to determine that precision is satisfactory.

(*2*) Add ca 100 mL extractant, then add the appropriate internal standard (IS) spiking solution [*see* Table **999.16C** and **C**]. Stopper flask with polyethylene stopper. (May stop overnight at this point.) That the volume of extractant is not exactly 100 mL is not important as long as the calculation is based on the volume of added internal standard; the order of addition of extractant and internal standard solution is important.

(*3*) Shake 1 h (shake by hand and release pressure before shaking mechanically). Secure the stopper with tape.

(*4*) Let large particles settle, and pour portion of extract into 50 mL centrifuge tube. (May stop overnight at this point.) May hold overnight at ca 4°C (to aid clarification) or at room temperature.

(*5*) Clarification is aided if extract is first chilled in ice bath for 30–60 min. Centrifuge 5 min at 2000 rpm (ca 1200 × *g*). Further dilute extract containing higher levels of analyte with diluent (as indicated in Table **999.16C**). Filter small portion through clarification filter, **B**(**g**), and inject.

F. Chromatography

(**a**) *LC–PCD configuration.*—

		mixing coil	detector	recorder
LC pump	LC column	**mixing tee**	pulse damper(s)	reagent pump

(**b**) *Chromatographic condition synopsis*.—Column: Spherisorb 5 µm, ODS2, 25 × 0.46 cm, or equivalent. Mobile phase: acetonitrile–2% HOAc [17 +83; *see* **C**(**g**)]. Flow rate: mobile phase 1.1 mL/min; PCD reagent, **C**(**p**), 0.6–0.7 mL/min (pump setting 1.25). Injection volume: 10 µL. Concentrated working standard (C_s): ca 5.5 µg SMT/mL. Detector: 450 nm, sensitivity 0.02 AUFS. Column conditioning: from the column stored in 100% organic solvent (acetonitrile or methanol), pump acetonitrile for a few minutes, then program to 17% acetonitrile in 2% acetic acid.

Table 999.16C Internal standard (IS) spiking solutions/ extract dilutions

Label claim SMT, mg/kg	IS[a] solution	IS solution, mL	Further dilution extract	D_u[b]
50–160	A	5.00	None	100
170–300	B	5.00	25/50	200
310–600	C	5.00	25/100	400
1100	Stock	5.00	10/100	1000
2200	Stock	10.00	10/200	2000

[a] *See* **C**, *Reagents*, for internal standard solutions.

[b] D_u = Total sample dilution used in calculation of result.

Adapt conditions as needed to satisfy the system suitability test.

(**c**) *LC–PCD system start-up*.—While pumping mobile phase, bring post-column system on-line. Let system equilibrate until baseline is satisfactory (10–30 min). (*Caution*: Wear gloves to protect skin. Reagent is strong acid and DMAB will discolor tissue.)

Optimum flow rates are 1–1.5 mL/min for the mobile phase, 0.5–1 mL/min for the post-column reagent; a mobile phase flow of 1.1 and reagent flow of ca 0.7 mL/min are satisfactory. Adjust as needed to obtain optimum baseline and analyte response.

Possible causes of baseline noise: (*1*) Reagent pulsations from PCD pump are not satisfactorily damped; (*2*) LC pump is not satisfactorily damped; (*3*) detector rise time is not properly set; (*4*) pump valves are leaking; (*5*) detector lamp is not stable (especially if H lamp is used); (*6*) air bubble is present in detector flow cell; (*7*) mixing coil is aged (fatigued); (*8*) mobile phase and post-column flow rates are not properly adjusted (with mobile phase at 1.3 mL/min, increasing the post-column reagent flow rate from ca 0.5 to ca 0.75 mL/min corrected a noisy baseline.)

(**d**) *Determination*.—*See* **D**, System Suitability Tests, for quality control standards. (*1*) Inject the system resolution standard solution, **D**(*1*), and adjust sensitivity/injection volume to produce peak height 50–80% full scale. Note whether resolution and peak shape are satisfactory. (Turnaround time is 12–20 min depending on the mobile phase used.) (*2*) Make 2 or more injections of working standard to ensure that peak response is repeatable (peak response relative standard deviation <2%). (*3*) Inject working standard followed by test injections. Bracket each set of 2 test injections by injections of working standard. In stable system, more tests may be injected between injections of standard. (*4*) Study chromatogram to determine if use of electronic measurement of peaks is justified (standard precision, peak integration marks, and baseline codes are satisfactory); otherwise, use manual peak height. (*5*) Calculate peak response ratios, SMT peak/SMR peak. If working standard response ratios throughout run are random, use average for calculation of results; if not random, average bracketing working standard ratios to calculate results.

(**e**) *LC column cleanup following analysis*.—Detach column from mixing tee, program from mobile phase to 100% aqueous, and hold for 20–30 mL of wash. Then program to 100% organic (about 10 min). Pass about 20–30 mL 100% organic through the column before shutting LC down and storing column in organic phase.

Oxalic acid solution, 0.2M, has been used successfully to rejuvenate columns in which resolution and peak shape have deteriorated. Program to water, pump 20–30 mL; pump 20–30 mL 0.2M oxalic acid; pump 20–30 mL water, and program to organic for storage of column.

(**f**) *Post-column system (including mixing coil and detector) cleanup following analysis of test set*.—After disconnecting LC column from mixing tee, plug the LC pump outlet in the tee, and pump methanol (ca 10 min) followed by isopropanol (10–15 min) through the system.

Methanol is the better solvent for cleaning reagent from tubing and detector; isopropanol is superior for seal protection.

G. Calculations

$$\text{Sulfamethazine, \%} = \frac{\times \quad \times \quad \times \quad \times}{\times \quad \times}$$

$$\text{Sulfamethazine, mg/kg} = \frac{\times \quad \times \quad \times \quad \times}{\times \quad \times}$$

where R = instrument response ratios; s = standard; W = weight in grams; u = unknown test portion; D = (total) dilution in mL; P = purity of standard.

Dilution calculation.—Total dilution of standard (D_s) in which W_s g standard material is dissolved in solvent, diluted to 100 mL, and further diluted in series 10/200 and 10/100 is calculated as:

$$D_s = \frac{100 \times 200 \times 100}{10 \times 10} = 20\,000 \text{ mL}$$

D_u = 100 mL or as indicated in Table **999.16C**.

Reference: *J. AOAC Int.* **83**, 260(2000).

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