RHÔNE·POULENC INC.
AGROCHEMICAL DIVISION

P O Box 125 - Black Horse Lane - Monmouth Junction, New Jersey 08852 - Telephone (201) 297-0100 - Telex: 844527

December 9, 1982
MAM/82/379

Richard Mountfort, PM #23
Environmetnal Protection Agency
Registration Division
Crystal Mall Bldg. 2
Arlington, VA 22202

Dear Mr. Mountfort:

Subject: EPA Reg. No. 359-662 Asulox Herbicide
PP# 6F1716 - alfalfa
PP# 6F1717 - pastures and rangeland
PP# 6F1766 - flax
Nonconfidentiality of methods submitted on April 15, 1982.

Rhone-Poulenc Inc. does not claim confidentiality of asulam methods numbered 154 (animal substrates) or 156 (plant substrates). The Environmental Protection Agency is therefore free to release these methods for tryouts.

Methods of ~~concern~~:

Rhone-Poulenc Method No. 154 - A common Moiety Method for the Measurement of Asulam and Its Principal Metabolites in Animal Biological Substrates by High Performance Liquid Chromatography.

Rhone-Poulenc Method No. 156 - A Common Moiety Method for the Measurement of Asulam and Its Principal Metabolites in Plant Substrates by High Performance Liquid Chromatography.

Sincerely,

RHONE-POULENC INC.
Agrochemical Division,

Margaret McMullen

Margaret A. McMullen
Registration Specialist

MAM/bjw

CONFIDENTIAL

PDD REPORT NO.: 81/003
REFERENCE NO.: 81/121/BHL/AG

RHÔNE-POULENC METHOD NO. 154

A COMMON MOIETY METHOD FOR THE MEASUREMENT OF ASULAM AND ITS PRINCIPAL METABOLITES IN ANIMAL BIOLOGICAL SUBSTRATES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

REPORTED BY: C. Guyton, Ph.D.
Senior Chief Chemist

APPROVED BY: A. Guardigli, Ph.D.
Manager
Analytical Services

RHÔNE-POULENC CHEMICAL COMPANY
AGROCHEMICAL DIVISION
Monmouth Junction, New Jersey

JUNE 1981

Rhône-Poulenc Chemical Company
Agrochemical Division
Monmouth Junction, New Jersey 08852

PDD REPORT NO.: 81/003

LAB REF NO.: 81/121/BHL/AG

Issue Date: June 1981

Analysis Conducted By:

Nick Somma Date 6/81
Nick Somma, B.S.
Senior Chemist

Date
Mark Piznik, B.S.
Research Chemist

Analysis Supervised and
Report Prepared By:

Charles L. Guyton Date 6/81
Charles L. Guyton, Ph.D.
Senior Chief Chemist
and Chemist-in-Charge

Report Approved By:

A. Guardigli Date 12/11/81
Alvaro Guardigli, Ph.D.
Manager
Analytical Services

Dates of Testing: 10/80 - 2/81

Rhône-Poulenc Chemical Company
Notebook No.: 1048, 1060, 1068, 1073

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I. ABSTRACT

A specific and sensitive method, suitable for enforcement purposes, is reported for measuring Asulam residues in animal substrates, based on a common moiety approach. All residues possessing the sulfanilamide nucleus are converted to N^4-acetylsulfanilamide. Following appropriate liquid-liquid and alumina column chromatography clean-up steps, acetylsulfanilamide is measured by high-performance liquid chromatography (HPLC) at 254 nm using a reverse phase column with 20% acetonitrile in pH 7.0 phosphate buffer as the mobile phase. The limits of detection for tissue, egg and milk samples are 0.05 ppm, 0.02 ppm and 0.01 ppm, respectively. Satisfactory recoveries were demonstrated for spiking levels from the limit of detection for each animal substrate to 25 ppm. A colorimetric thin-layer chromatography confirmatory test is provided. In addition, results from an interference study show that some 19 tolerance-containing pesticides and sulfa drugs do not interfere with Asulam residue determinations.

II. INTRODUCTION

Methyl-4-sulfanilylcarbamate (Asulam) is a selective herbicide active both pre- and post-emergence against a wide range of broadleaf and grassy weeds. It is particularly effective post-emergence for the control of tansy ragwort, bracken fern, dock, johnsongrass, and other important weed species in such crops as sugarcane, flax, alfalfa, pastures and non-crop situations.

Since some of the agricultural products treated with Asulam are used as animal feed, it is necessary to have an analytical method for measuring Asulam residues in animal tissue and animal by-products, such as milk and eggs.

The results (Heijbroek, W. H. M., 1972) of an Asulam (^{14}C-UL ring) rat metabolism study showed that at least 90% of the orally administered compound was eliminated within twenty-four hours. The components isolated from the urine were mainly Asulam (86%) and 4-acetylasulam (12%). An Asulam (^{14}C-UL ring) metabolism study in lactating goats has been reported by Unsworth (Unsworth, J.B.; 1975). Goats received the herbicide in the diet corresponding to a 100 ppm feeding level. Three days after dosing, 62-71% of the administered dose was excreted in the urine and about 20% was excreted in the feces. An essentially quantitative accountability (91-102%) was achieved seven days post-dosage. Expired gases accounted for only a small portion of the dose, i.e., <0.5%. Only about 0.1% of the dose was excreted in the milk. Some goat radioactivity was associated with naturally occuring chemicals. The urinary radicactivity consisted mainly of parent compound and acetylasulam, just as in the rat study. Compounds isolated from tissues and milk were Asulam, acetylasulam, and acetylsulfanilamide. See Figure 1 for the structural formulae for these compounds and sulfanilamide, a related chemical.

The above results of Unsworth were corroborated in a related lactating goat study (ADC, 1981a) which served as a source of ^{14}C-Asulam treated tissue and milk samples for conducting a analytical method validation study. The animal received multiple doses of 276 ppm of Asulam in order to obtain "aged" residues. The goat study demonstrated that the radioactivity could be extracted most efficiently from tissues using 1N hydrochloric acid. The extractable tissue and milk residues were characterized by thin-layer chromatography (TLC) as unchanged parent compound, acetylasulam, 4-acetylsulfanilamide and sulfanilamide. These chemicals accounted for essentially all the residue present in the animal extracts.

In the method validation study (ADC, 1981b), ^{14}C-treated samples were processed through the analytical method. Residue determinations by radiometric assay and HPLC were compared, and good agreement was found between the two analytical techniques.

Since more bound ^{14}C material was found in the liver sample than the other tissue samples, a test (ADC, 1981b) was made to determine if the bound material represented a sizeable Asulam equivalent residue. Following the extraction procedure in the method, the liver filter cake was subjected to an acid hydrolysis treatment which liberated about one-half of the bound ^{14}C material. This filtrate was then processed through the method. The results showed that this extra treatment did not add substantially to the common moiety residue picture even though more ^{14}C material was extracted initially.

III. PRINCIPLE OF THE METHOD

Asulam and its metabolites; acetylasulam, sulfanilamide, acetylsulfanilamide and any free conjugate containing the sulfanilamide moiety, were extracted from animal samples by a hydrochloric acid/acetonitrile mixture. An acetylation reaction on the N^4 group converted Asulam and the sulfanilamide moieties to their respective acetylated derivative. Next, a mild acid hydrolysis was employed to hydrolize any carbamate function from the acetylated asulam and from the metabolite acetylasulam to acetylsulfanilamide. After appropriate clean-up steps, the total residue present was measured by high performance liquid chromatography equipped with an ultra-violet detector and reported as Asulam equivalents by comparing the samples response against a calibration curve of standard N^4-acetylsulfanilamide, expressed in terms of Asulam.

IV. DISCUSSION OF THE METHOD

This validated, specific method describes the analytical procedures employed to measure "total Asulam" residues in animal tissues, milk and eggs. The limits of detection for egg, milk and tissue are 0.02 ppm, 0.01 ppm and 0.05 ppm, respectively. Schematic diagrams for processing egg/tissue and milk samples are shown in Figures 2 and 3, respectively.

Recovery data were generated by adding known amounts of Asulam, acetylasulam, 4-acetylsulfanilamide and sulfanilamide, as individual chemicals and in combinations, to untreated samples at the homogenization step and then processing the samples through the procedure. All recovery data for the chemicals are based on Asulam equivalents. The recovery data for egg samples are given in Table 1. The spiking levels ranged from 0.02 to 5.0 ppm. The average recovery was 88.6% with a standard deviation of 7.3. In Table 2 are shown recovery data for milk spiked at 0.01 to 0.1 ppm with an overall recovery of 73.0%. The standard deviation was 5.6. Animal tissue recovery data are presented in Table 3. The grand mean recovery was 83.5 with a standard deviation of 7.5 for samples spiked at 0.05 to 25 ppm.

It was determined that the radial compression unit for HPLC offered advantages over the conventional rigid metal column. The conditions for manual and automatic operations of the HPLC are given in Section VI of this method. Standard calibration curves for 4-acetylsulfanilamide expressed as Asulam equivalents are shown as Graph 1 and Graph 2 under manual (peak height) and automatic (peak area) conditions, respectively. The automatic operations offer a decided advantage in that the concentration of the standard is linear with respect to peak area from 0.1 to 100 µg/ml.

A confirmation test for detecting 4-acetylsulfanilamide was developed. The solution containing the chemical was spotted on a silica gel plate and the plate developed in a toluene/glacial acetic acid (1:1) mixture. The 4-acetylsulfanilamide spot was visualized by first spraying with acid followed by a heat treatment to convert the chemical to an aryl-amine. The classical Bratton-Marshall reagent was then sprayed on the plate. Nice magenta spots appeared. See Figure 4 (RS-49) for standards from 2.0 to 0.1 µg.

Some typical values for control (untreated) samples are given in Table 4. These samples were taken through the method and any response on the chromatogram was calculated as apparent Asulam equivalent residues. These values were less than the limit of detection.

An interference study was conducted on some 19 herbicides, insecticides, fungicides and sulfa drugs that might be found in conjunction with Asulam and its metabolites in egg, milk and meat. These chemicals were processed through a partial procedure and examined by HPLC. Retention time and response data are presented in Table 5. Graph 3 shows results of some sulfa drugs that were taken through the entire procedure.

Some data on the completeness of hydrolysis and acetylation along with some extraction tests are given in Appendix II. These data were generated using standards of Asulam, acetylasulam, 4-acetylsulfanilamide and sulfanilamide without the presence of biological substrates. Some data on the method validation study and HPLC data of acetylated sulfanilamide are also included in Appendix II.

V. EXPERIMENTAL ANALYTICAL PROCEDURE FOR DETERMINING TOTAL ASULAM EQUIVALENT

A. CHEMICALS/REAGENTS - EQUIPMENT - REFERENCE STANDARDS:

1. Chemicals/Reagents

Sodium nitrite - USP, Fisher Scientific
Glacial acetic acid - Reagent, Baker, J.T. Baker
N-1-Naphthylethylene diamine dihydrochloride - Eastman Kodak; Rochester, N.Y.
Hyflo Super Cel - Fisher Scientific
Acetic anhydride - A.C.S. Certified, Fisher Scientific
Sodium acetate anhydrous - A.C.S. Certified, Fisher Scientific
Aluminum sulfate - A.C.S. Certified, Fisher Scientific
Deionized water
Organic solvents (pesticide grade, glass distilled solvents) - Burdick & Jackson Labs, Inc.; Muskegon, Michigan
pH7 Buffer Concentrate - Certified, Fisher Scientific
Hydrochloric acid - A.C.S. Reagent, Fisher Scientific
Sodium hydroxide - A.C.S. Certified, Fisher Scientific
Sodium sulfate anhydrous - Reagent grade, granular, Fisher Scientific
Aluminum oxide for chromatography - Acid; Brockmann Activity I; Camag
Glass Wool
Dry Ice
Glass fiber filter discs - Reeve Angel; Clifton, New Jersey

2. Equipment

Centrifuge Tube - 100 ml, 250 ml, Sorvall
pH meter - Fisher Acumet® Model 142, Single electrode
Assorted laboratory glassware
Mechanical shaker "Atlab" or equivalent; Arthur Thomas Co.; Philadelphia, Pa.
Rotary Vacuum Evaporator - "Buchler" or equivalent (all glass system)
Glass column for chromatography - mini column; size C, O.D. 12 mm, length 300 mm (Kromflex; Kontes, Vineland, N.J.)
TLC plates - Silica gel glass plate, 20x20 cm; pre-coated, 60 F-254, E. Merck Co.
Waring blender or equivalent - explosion-proof
Polytron - PT10/35 homogenizer, PT20ST probe, Brinkman Instruments
Water Baths - Fisher Versa-Bath (or equivalent)

High Performance Liquid Chromatograph (HPLC) - Waters Associates (See HPLC section)
Analytical Balance - Mettler, H-8
Centrifuge - Sorvall GLC-1
Heating Mantle - six unit manifold, Glas-Col Apparatus, K-J 500, Terre Haute, Ind.
Oven - Lab-line Imperial II, Lab-line Instruments, Inc., Melrose Park, Illinois

3. Reference Standards

The following anlaytical reference standards were made available by May & Baker Ltd., England, member of the Rhone-Poulenc group of companies.

Compound	Lot No.	Purity %
Asulam	LOP 1770	99.9
N^4-Acetylasulam	KWC 1002	98.7
N^4-Acetylsulfanilamide	KWC 997	99.9

(hereafter referred to as 4-acetylsulfanilamide or simply acetylsulfanilamide)

A commercial house provided the standard sulfanilamide (Fisher Certified Reagent of 98% minimum purity).

B. SAMPLE PREPARATION AND HOMOGENIZATION

1. Tissues - Meat and Meat By-Products

Dice semi-thawed samples into cubes (1-2 cm.) suitable for grinding in a Waring blender and in the presence of an equal volume of dry ice. Reduce sample to coarse small pieces and allow dry ice to sublime. Weigh a 10 gram representative sub-sample of minced tissue into a 100 ml. centrifuge tube. Add 25 ml. each of 1N hydrochloric acid and acetonitrile and blend for about two minutes at middle speed using the Polytron homogenizer.

2. Eggs and Blood

Weigh a 10 gram representative sub-sample of whole blood or whole eggs into a 100 ml. centrifuge tube. Add 25 ml. each of 1N hydrochloric acid and acetonitrile. Mix the sample well by blending for about 30 seconds using the Polytron at the number 1 setting (slow speed).

3. Whole Milk

Weigh a 50 gram representative, thoroughly mixed sub-sample of whole milk into a 250 ml. centrifuge tube. Add 25 ml. each of 1N hydrochloric acid, acetonitrile and methanol and 10 grams of Hyflo Super Cel. Blend with the Polytron for two minutes at middle speed.

C. CENTRIFUGATION AND FILTRATION

Cap the centrifuge tubes containing the homogenates of tissues, milk or eggs; then spin the sample at 3000 rpm for five minutes. Filter the supernatant liquid through a Buchner funnel, fitted with two pieces of 7 cm. glass fiber filter discs, into a 250 ml. filter flask under reduced pressure. Repeat the extraction, homogenization, centrifugation and filtration steps again. Transfer the combined filtrates into a 500 ml. boiling flask and evaporate until solvent free with the aid of a rotary vacumm evaporator. Bath temperature not to exceed 40°C.

D. PRECIPITATION CLEAN-UP

Add 10 ml 25% aluminum sulfate solution to the aqueous sample. Adjust the pH to between 4 and 7 with ~ 6 ml 10 N NaOH using a pH meter. Allow the sample to cool and filter through a Buchner funnel fitted with two pieces of

7 cm. glass fiber filter discs into a 250 ml filter flask under reduced pressure. Wash the filter cake with ∿ 15 ml deionized water. Transfer the filtrate quantitatively to a 500 ml boiling flask.

E. ACETYLATION

Add 3 gm sodium acetate and 5 ml acetic anhydride to the sample. Cap the flask tightly and mechanically shake for one hour at room temperature.

F. HYDROLYSIS

Adjust the pH to 3.0 with ∿4 ml of 5 N HCl using a pH meter. Connect the flask to a water cooled condenser and place in a heating mantle. Allow the sample to reflux gently for one hour. Remove the flask from the heating mantle immediately after the reflux time and allow the sample to cool to room temperature. Rinse the condenser with a few ml of deionized water. (Note: Do not allow the sample to stand for a prolonged period of time after this step.)

G. LIQUID-LIQUID PARTITION CLEAN-UP

a. Ethyl Acetate Extraction

Transfer the hydrolysate to a 500 ml separatory funnel and extract 1x100 ml hexane and discard the hexane wash. Extract the aqueous phase 3x equal volumes of ethyl acetate (∿ 150 ml) and filter each ethyl acetate extraction through anhydrous sodium sulfate into a 1000 ml boiling flask. Evaporate the organic solution to near dryness using a rotary vacuum evaporator. At this point, a small amount (∿ 4 ml) of acetic acid is usually present in the flask; add ∿ 100 ml acetonitrile and re-evaporate to remove the trace amounts of acid. If acid remains, repeat addition of acetonitrile until all traces of acetic acid are gone. Bath temperature not to exceed 40°C.

b. Milk Clean-Up Step

Transfer the oily contents of the flask following the evaporation of ethyl acetate to a 500 ml separatory funnel using 100 ml acetonitrile in several rinses. Add 100 ml hexane to the separatory funnel and shake. Discard the hexane. Add the acetonitrile to a 500 ml boiling flask and remove the solvent with the aid of a rotary vacuum evaporator. Remove any acetic acid as described above. Bath temperature not to exceed 40°C.

H. ALUMINA COLUMN CHROMATOGRAPHY

Place a plug of glass wool in the bottom of a 300 x 12 mm O.D. chromatographic tube having a coarse sintered glass disc.

Pour a suspension of 10 gm of aluminum oxide acid Brockmann Activity I (Camag) in ethyl acetate into the column. Allow the material to settle and place a plug of glass wool at the top. Do not allow the column to go dry at any time. Wash the column with an additional 25 ml of ethyl acetate.

Transfer the contents in the boiling flask to the column with a small portion of ethyl acetate. Continue to rinse the sample flask with small portions of ethyl acetate until 50 ml has been used and discard the eluate.

Continue the elution with 35-40 ml of 1% methyl alcohol in ethyl acetate and discard.

Elute and collect 100 ml 2% methyl alcohol in ethyl acetate in a 250 ml boiling flask.

Evaporate the solvent to dryness and bring the sample to appropriate final volume with 20% acetonitrile in buffer pH 7.0 for HPLC quantitation.

Bring up tissue, egg and milk samples in 5, 2 and 5 ml, respectively, of mobile phase.

Note 1. The activity of the alumina at hand was confirmed by running known amounts of standard acetylsulfanilamide through the column. Appropriate aliquots of the eluate were quantitated on HPLC to determine the elution scheme.

Note 2. Samples can be safely stored overnight preferably in a refrigerator, at several points in the procedure. These are: 1) after the precipitation clean-up; 2) after acetylation; 3) after ethyl acetate extraction; 4) after the alumina column clean-up.

VI. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

A. HPLC INSTRUMENT OPERATING PARAMETERS

INSTRUMENT	- Waters Associates HPLC
MANUAL MODE	- U6K Injector
DETECTOR	- 450 Variable Wavelength UV
PUMP	- Model 6000-A
COLUMN	- Radial - Pak C18 8mm x 10cm for RCM - 100 Radial Compression Module
OPERATING PARAMETERS	- Mobil Phase: 20% acetonitrile in pH7 - Flow Rate: 1 ml/min. - Wavelength: 257 nm; Attenuation 0.04AUF - Injection Volume: 80 µl - Chart Speed: 0.5 cm/min.
AUTOMATED MODE	- WISP 710 - B autoinjector - Data Module 730 - System Controler 720
DETECTOR	- 440 UV
PUMP	- Model 6000-A
COLUMN	- Radial - Pak C18 8mm x 10cm for RCM - 100 Radial Compression Module
OPERATING PARAMETERS	- Mobil Phase: 20% acetonitrile in pH7 - Flow rate: 1 ml/min. - Wavelength: 254 nm;Attenuation 0.02AUF - Injection Volume: 45 µl - Run Time 8 minutes. Delay time 25 min. - Two injection of standard; one of sample - Chart speed 0.5 cm/min.; LC Mode - Report format: Waters Associates 8801 (WISP)
CALIBRATION/INTEGRATION	- Peak Identification: N^4-Acetyl-sulfanilamide - Retention time:5.7 min.;Peak Width: 12 - Noise Rejection: 25 - Area Rejection: 50,000 - Retention time window ± 6% - Inhibit Integration: 0.1; 7.0 min. - Resume Integration: 5.0 min. - Report format 33 (001000-Waters) not reporting unknowns (Data Module)

B. PREPARATION OF LINEARITY CURVE

Accurately weigh 0.0935 grams of standard acetylsulfanilamide using an analytical balance into a 100 ml volumetric flask.

Dissolve the contents with methanol and make graded serial dilutions of the stock solution to give working standards of 1 µg/ml to 0.1 µg/ml of Asulam equivalents of the standard. Protect the solutions from light.

For manual calculations, inject the appropriate amount of acetylsulfanilamide standard for concentrations from 0.1 to 1.0 µg/ml. Plot concentration vs. peak height (mm). See Graph 1.

For automatic calculations, inject 45 µl of acetylsulfanilamide standards for concentrations from 0.1 µg/ml to 150 µg/ml. See Graph 2 for the calibration curve covering concentrations from 0.1 to 100 µg/ml plotted vs. peak area.

C. SAMPLE DETERMINATION

1. Manual

Make a suitable µl injection of standard solution C, µg/ml, to achieve 100-140/mm peak height followed by an injection of the same volume for samples. If any sample response is outside the linear dynamic range of the standard curve, dilute the sample with mobile phase until the response falls within range of the calibration curve. The peak height of the sample (h) is compared to the peak height of the standard (H). The calculation is made as follows:

$$\frac{h}{H} \times C = \mu g/ml \text{ of sample}$$

$$\mu g/ml \text{ of sample} \div \frac{\text{weight of sample,g}}{\text{volume of sample,ml}} = \text{ppm}$$

$$\text{ppm corrected} = \text{ppm} \times \frac{100}{\text{percent recovery}}$$

2. Automatic

Inject the same volume of sample as the standard (ca. 45 µl). An external standard method* with calibration averaging was used. Using the scale factor, corrected ppm values are given. The system automatically rejects any area response less than 50,000 and it does not calculate any peak response unless the peak is at the proper retention time.

Response factor (Rf) at retention time:

$$Rf = \frac{\text{standard amount (ppm)}}{\text{area of peak}} \times 1000$$

Where ppm=standard conc.÷ sample dilution(g/ml)

Sample response at retention time**

$$\text{Amount (ppm)} = \frac{Rf\ (\text{standard})}{1000} \times \text{area of sample peak} \times \text{correction factor}^{***}$$

* Data Module Instruction Manual, External Standard Method; p. 5-49 and 5-53

** Retention time may vary up to 6% of standard

*** For treated samples only: $\frac{100}{\%\ \text{Recovery}}$

Example Calculation:

Egg No. 2968 sp. 0.1 ppm Asulam
Dilution: 10 g./2 ml

Standard Calibration Averaging

#1 Rf = $\frac{0.20\ \text{ppm}}{1121485} \times 1000 = 0.178300 \times 10^{-3}$ (E-3)

#2 Rf = $\frac{0.20\ \text{ppm}}{1146352} \times 1000 = 0.174400 \times 10^{-3}$ (E-3)

Average = 0.176300×10^{-3} (E-3)

Peak area = 456100

Sample Amount = $\frac{0.176300\ \text{E-3}}{1000} \times 456100 = 0.0804$ ppm

Recovery = $\frac{0.0804}{0.1000} \times 100 = 80.4\%$

The sequence of events for the Data Module 730 print-out are a HPLC chart with stated retention time (minutes), system controller conditions, sample identification, WISP information including sample position and injection volume, method of quantitation and data results, including peak identification, corrected ppm (using scale factor), retention time, area and Rf.

VII. CONFIRMATORY PROCEDURE BY THIN-LAYER CHROMATOGRAPHY

Take a suitable aliquot of the 2% methanol/ethyl acetate eluant at the alumina column clean-up step, and evaporate the solvent with a rotary vacuum evaporator. Dissolve the residue with an appropriate volume of methanol. Spot and proceed as directed for standard acetylsulfanilamide, as given below:

Standard 4-Acetylsulfanilamide (See Figure 4)

Suitable dilutions of the working standard solutions were made, and 0.1, 0.2, 0.3 0.5, 1.0 and 2.0 µg amounts of acetylsulfanilamide were applied about 2 cm from the bottom of a TLC plate. The TLC plate (used "as is") was then developed, by the ascending technique, for about 14 cm in a well-equilibrated solvent tank (no blotter paper) containing a glacial acetic acid/toluence (50:50, v/v) mixture.

After development, the plate was completely dried under a hood. A preliminary examination of the plate was made under short-wave UV light; however, this method of detection was not sensitive enough. Acetylsulfanilamide was easily visualized, however, through the formation of the corresponding azo-dye. Acetylsulfanilamide was hydrolyzed to an aromatic amine by first heavily spraying the plate, after development and drying, with a mixture of hydrochloric acid/ methanol (50:50). The plate was placed in a 110°C oven for 45 minutes. After cooling, the plate was again lightly sprayed with the hydrochloric acid/methanol mixture. Then a freshly prepared methanolic solution of 2% sodium nitrite (prepared by dissolving two grams of chemical in a few milliliters of water before adding methanol to the 100 ml mark) was sprayed lightly on the plate two times, allowing the plate to dry after each application.

Finally, 1.0% Bratton-Marshall Reagent (one gram of chromogenic reagent was dissolved in 5 ml water, 2 ml concentrated hydrochloric acid and diluted to volume with methanol) was sprayed uniformly on the plate. Bright magenta spots appeared on a white background. Under typical conditions, the compound has the following Rf values:

Reference Standard Spot	Rf
Acetylsulfanilamide	0.18

VIII. VALIDATION OF THE METHOD

The validity of the described analytical procedure for total Asulam was tested by adding known amounts of Asulam, acetylasulam, acetylsulfanilamide and sulfanilamide to control (untreated) samples at the homogenization step and processing the spiked samples through the entire procedure. These recovery data are reported in Tables 1 - 3 and demonstrate that satisfactory recoveries can be achieved.

In addition, control samples of egg, tissue and milk were processed through the procedure and analyzed for any "apparent" Asulam residues. Any peak response that corresponded to the appropriate retention time for Asulam equivalents was quantitatively estimated. These values for milk ranged from no peaks to 0.003 ppm, all below the limit of detection, 0.01 ppm.

Control tissue values were mostly clean at the proper retention time or only marginal responses were seen, i.e., <0.05 ppm. The limit of detection for tissue samples is considered to be 0.05 ppm. The limit of detection for egg is 0.02 ppm, based on the observation that control egg samples were clean at the proper retention time. Table 4 gives apparent residue values for control samples.

When the method was "validated" by processing "aged" ^{14}C-tissue through the various steps in the analytical procedure, there was good agreement between HPLC and radioassay results, as shown in Appendix II, specimen Table 9 "Comparison of HPLC and LSC Data from ^{14}C-Goat Samples" (A.D.C., 1981 b). Fat, kidney, muscle, liver and milk samples were investigated.

IX. INTERFERENCE STUDY

Selected herbicides (4), insecticides (7), fungicides (1), and sulfa drugs (7) with tolerances in milk, eggs and/or meat and meat by-products were examined by the Asulam method. Compounds were selected based on structure and tolerance level. Standards were prepared and 5 µg of each compound was passed through the acetylation, hydrolysis and extraction steps in the analytical procedure. Samples were brought to volume in mobile phase (5 ml), giving the concentration of 1 µg/ml, and were injected into the HPLC.

Comparison of sample chromatograms with an acetylsulfanilamide 1 µg/ml standard chromatogram showed no significant interference at the appropriate retention time with the exception of sulfaquinoxaline, sulfathiazole, and sulfadimethoxine. Standards of these three compounds were processed through the entire procedure, including the alumina column. The interfering peak was completely removed in the sulfathiazole sample, but only reduced for the sulfaquinoxaline and sulfadimethoxine samples. See Graph 3.

However, if these two sulfa drugs were present in tissue at their 0.1 ppm tolerance level, they would contribute to the Asulam residue about 0.05 ppm, or the limit of detection.

The data are summarized in Table 5. The retention times and peak responses are stated relative to acetylsulfanilamide.

X. LITERATURE REFERENCES

Heijbroek, W. H. M. (1972): Scientific Report from The Research Laboratory of May & Baker, Ltd.; Dagenham, England (Confidential communication RG/1425- Submitted to EPA 11/6/72; Absorption, Excretion and Metabolism in the Rat

Unsworth, J. B. (1975): Scientific Report from The Research Laboratory of May and Baker, Ltd.; Dagenham, England (Confidential communication RES/2359 - Submitted to EPA 12/15/75; Metabolism in Goats

ADC (1981a): Scientific Report from Analytical Development Corporation; Monument, Colorado (Confidential communication) ADC Project #517 (^{14}C-Asulam Goat Study)

ADC (1981b): Scientific Report from Analytical Development Corporation; Monument, Colorado (Confidential communication) ADC Project #517-B (Validation of Method No. 154 by Radiometric and Conventional Means)

APPENDIX I

Table 1

RECOVERIES FOR ASULAM AND ITS METABOLITES IN EGG

Substrate Description	Spiking Levels* ug	ppm	Chemicals**	Percent Recovery *	Remarks
Whole Egg	50.0	5.00	A	79.8	
	10.0	1.00	A	95.2	
	10.0	1.00	A, AA, AS, S	79.8	(0.25 ppm each)
	5.0	0.50	AS	93.7	
	5.0	0.50	A	96.0	
	4.0	0.40	A, AA, AS, S	98.6	(0.1 ppm each)
	1.6	0.16	A, AA, AS, S	88.8	(0.04 ppm each)
	1.0	0.10	A	80.4	
	0.6	0.06	AA, AS, S	87.8	(0.02 ppm each)
	0.2	0.02	A	77.3	
	0.2	0.02	AA	89.1	
	0.2	0.02	S	96.1	
	0.2	0.02	AS	89.5	
			Mean	88.6	
			Standard Deviation	7.3	

* Asulam equivalents

** A = Asulam AS = Acetylsulfanilamide
AA = Acetylasulam S = Sulfanilamide

TABLE 2

RECOVERIES FOR ASULAM AND METABOLITES IN MILK

Substrate Description	Spiking Levels* ug	ppm	Chemicals**	Percent Recovery *	
Whole Milk[a]	5.0	0.10	A	71.6	72.4
	5.0	0.10	AA	63.4	73.0
	5.0	0.10	AS	76.2	78.0
	5.0	0.10	S	78.8	78.8
	0.5	0.01	A	64.4	64.4
	0.5	0.01	AA	74.0	75.4
	0.5	0.01	AS	80.0	68.6
	0.5	0.01	S	70.4	79.2
			Mean	73.0	
			Standard Deviation	5.6	

* Asulam equivalents

** A = Asulam
AA = Acetylasulam
AS = Acetylsulfanilamide
S = Sulfanilamide

a Data generated by A.D.C.

TABLE 3 RECOVERIES FOR ASULAM AND METABOLITES IN TISSUES

Total Spiking Levels* pg	ppm	Chemicals **	Substrate/Recovery % Muscle White	Muscle Dark	Fat/Skin	Kidney	Liver	Heart	Gizzard	Whole Blood	Remarks
250.0	25.0	A				79.4					
200.0	20.0	A, AA				90.9					10 ppm ea.
100.0	10.0	A, AA					74.2				5 ppm ea.
80.0	8.0	A, AA, AS, S				91.1					2 ppm ea.
50.0	5.0	A	83.9	84.0		90.0	85.5		75.9		
20.0	2.0	A	84.2	85.3			92.0				
20.0	2.0	A, AA, AS, S	86.0			91.1					0.5 ppm ea.
20.0	2.0	A, AA, AS, S					80.9				0.4 ppm ea. except A,0.8
10.0	1.0	A	80.7	88.9		88.9	91.1	91.0	76.3	77.6	
8.0	0.8	A, AA, AS, S	85.1								0.2 ppm ea.
5.0	0.5	A	83.5	74.4	85.4	86.3					
5.0	0.5	AA		71.5							
5.0	0.5	AS		86.2							
5.0	0.5	S			90.4						
5.0	0.5	A, AA, AS, S					63.5				0.1 ppm ea. except A, 0.2
4.0	0.4	A, AA, AS, S	77.6		67.8				94.7	86.0	0.1 ppm ea.
2.0	0.2	A			93.3						
2.0	0.2	AA			83.4						
2.0	0.2	A, AA, AS, S						91.1			
1.0	0.1	A	87.3	82.5	89.5			93.9	80.3	78.8	
1.0	0.1	A, AA			88.3						0.05 ppm ea.
0.5	0.05	A	76.6		83.4		70.0	79.7			
0.5	0.05	A, AA			65.6						0.025 ppm ea.
0.5	0.05	AS, AA					80.8				0.025 ppm ea.
0.5	0.05	A, AA, AS, S				82.8					
0.5	0.05	A, AA, [illegible] S					89.6				0.0125 ppm ea.

Grand Mean = 83.5

TABLE 4

APPARENT ASULAM RESIDUES IN CONTROL EGG, TISSUES AND MILK SAMPLES

Sample Description	Apparent Asulam Residues (ppm*)
Milk	ND**, 0.002 0.002, 0.003
Heart	ND, ND
Kidney	<0.01, <0.01
Blood	<0.01 <0.01
Liver	ND, ND
Gizzard	ND, <0.01
Skin/Fat	ND, ND
Muscle, dark	ND, ND
Muscle, white	<0.01, ND
Egg	ND, ND, ND

*ppm - Based on peak height

**ND - Denotes no peak at proper retention time

TABLE 5

RETENTION TIME AND RESPONSE OF SELECTED PESTICIDES AND SULFA DRUGS PROCESSED THROUGH ACETYLATION AND HYDROLYSIS STEPS

Compounds (µg/ml)	Response*	Relative Retention Time
Standard Acetylsulfanilamide	100%	1.00**
Sulfanilamide		0.87
Asulam & Acetylasulam		0.51
Herbicides:		
Terbacil	Weak	1.23
2,4-D	Weak	1.14
Tordon	ND	ND
Metribuzin	ND	ND
Insecticides:		
Carbaryl	ND	ND
Chlordimeform	ND	ND
Diazinon	Weak	0.69
Malathion	ND	ND
Chlorpyrifos	Weak	1.26
Zolone	Weak	1.23
Fungicides:		
Benomyl	Strong	0.39
Acaricide:		
Chlorobenzilate	ND	ND
Sulfa Drugs:		
Sulfaquinoxaline	Moderate	1.04
Sulfamethazine	ND	ND
Sulfapyridine	ND	ND
Sulfathiazole	Strong	1.12
Sulfamethoxypyridazine	Strong	1.24
Sulfadimethoxine	Moderate	1.00
Sulfaguanidine	Moderate	0.88

* Strong - 70-100% of standard
Moderate-40- 70% of standard
Weak - 0- 40% of standard
ND - None detected

** Retention time 5-6 minutes

RESPONSE FOR SULFAQUINOXALINE, SULFATHIAZOLE AND SULFADIMETHOXINE PROCESSED THROUGH THE COMPLETE TISSUE PROCEDURE

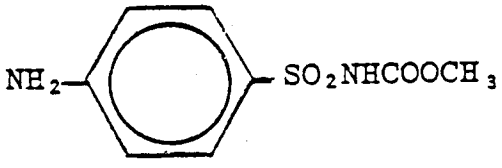
Chemical (5 ug each***)	Dilution	Response ppm
Sulfaquinoxaline	10g/5ml	0.24
Sulfadimethoxine	10g/5ml	0.25
Sulfathiazole	10g/5ml	None

*** 5 times tolerance level

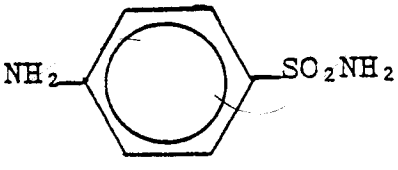
Figure 1

STRUCTURAL FORMULAE

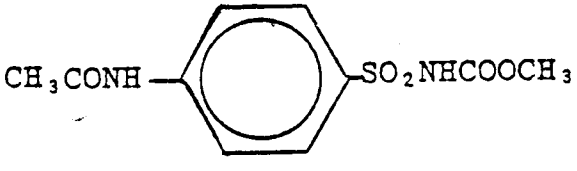
Methyl-4-sulfanilylcarbamate



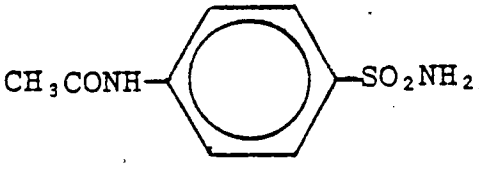
ASULAM
(M&B 9057)



SULFANILAMIDE
(Fisher Certified)



N^4-ACETYLASULAM
(M&B 9495)



N^4-ACETYLSULFANILAMIDE
(M&B 24805)

Figure 2

SCHEMATIC PROCEDURE DIAGRAM
FOR EGG/TISSUE

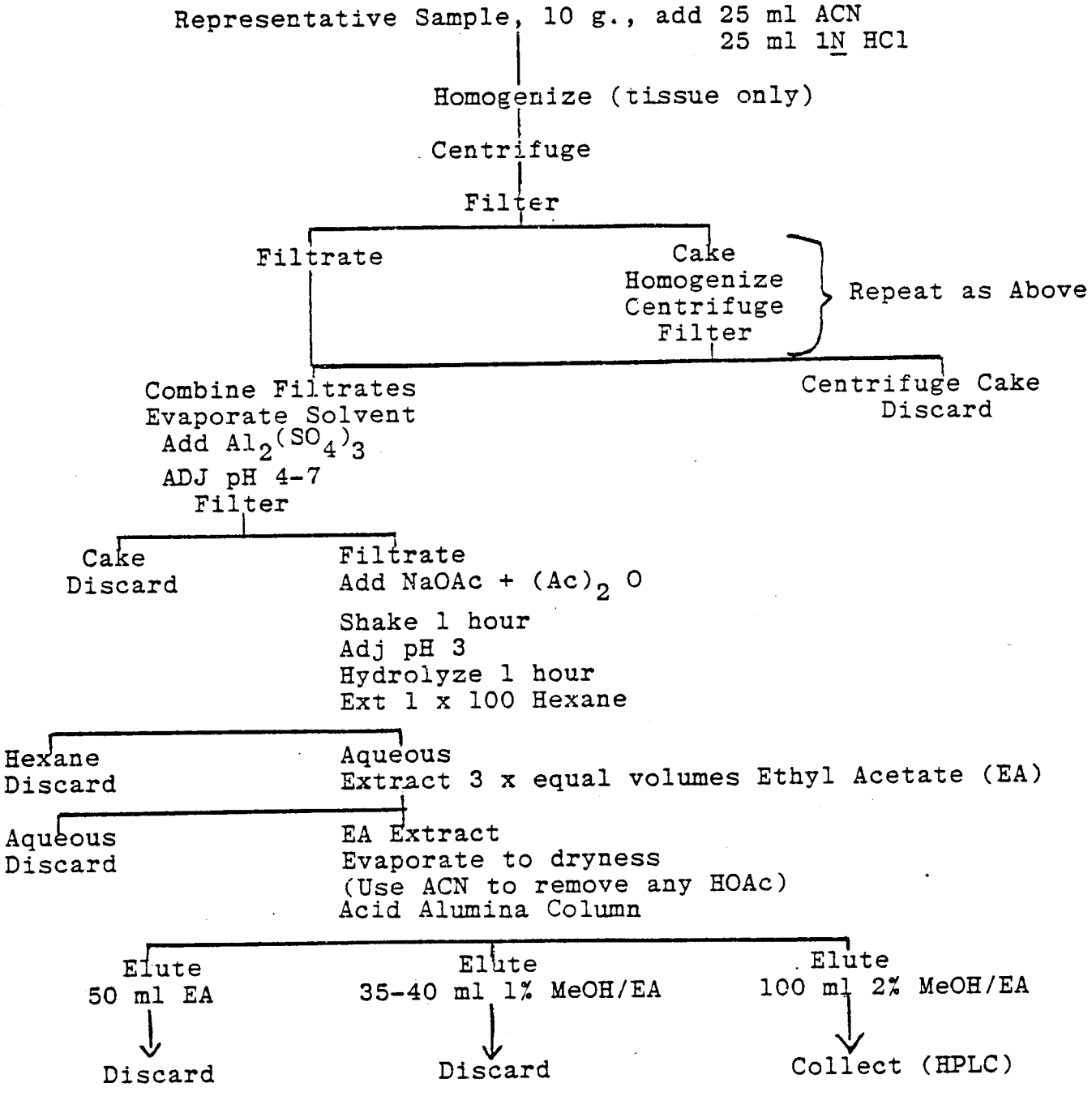


Figure 3

SCHEMATIC PROCEDURE DIAGRAM
FOR MILK METHOD

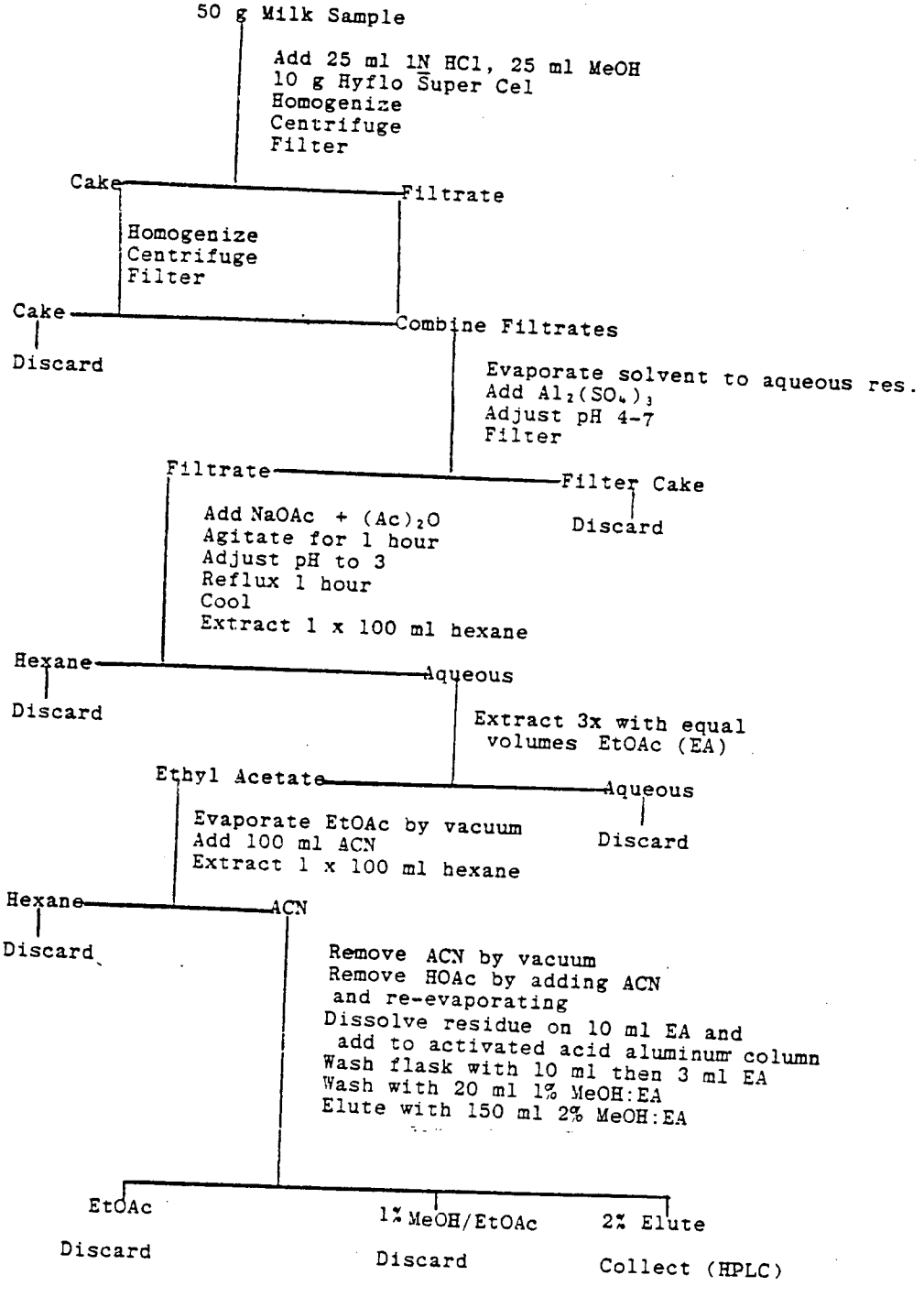


Figure 4

PHOTOGRAPH RS-49

CONFIRMATION TEST FOR ACETYLSULFANILAMIDE

Acetylsulfanilamide

R_f = 0.18

Channel	Description
1	0.1 µg reference standard
2	0.2 µg reference standard
3	0.3 µg reference standard
4	0.5 µg reference standard
5	1.0 µg reference standard
6	2.0 µg reference standard

Developing Mixture: Toluene/Glacial Acetic Acid (1:1;V,V)

Visualization: Bratton-Marshall Reagent after acid treatment as described earlier

GRAPH 1

ACETYLSULFANILAMIDE

CALIBRATION CURVE

Manual Operation

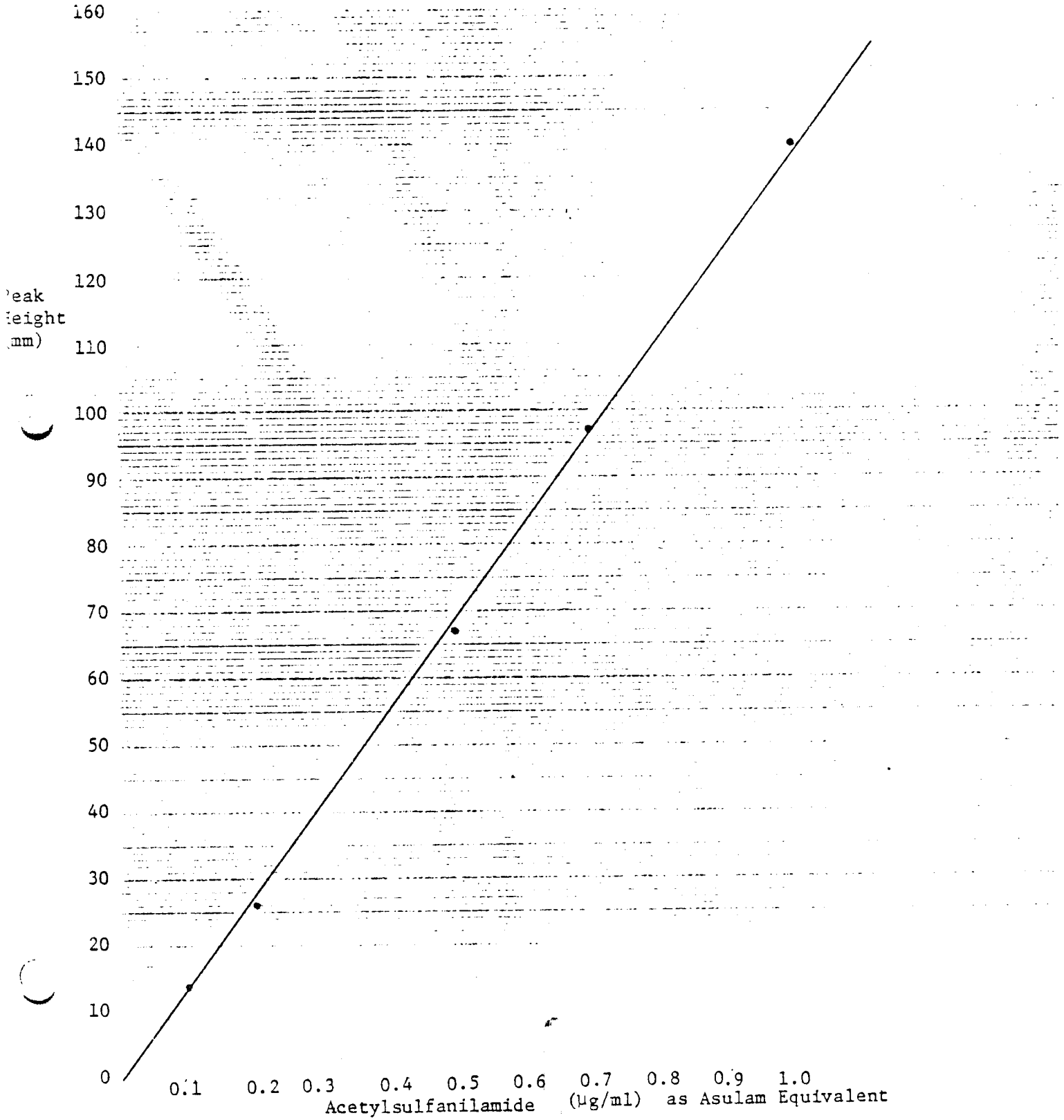
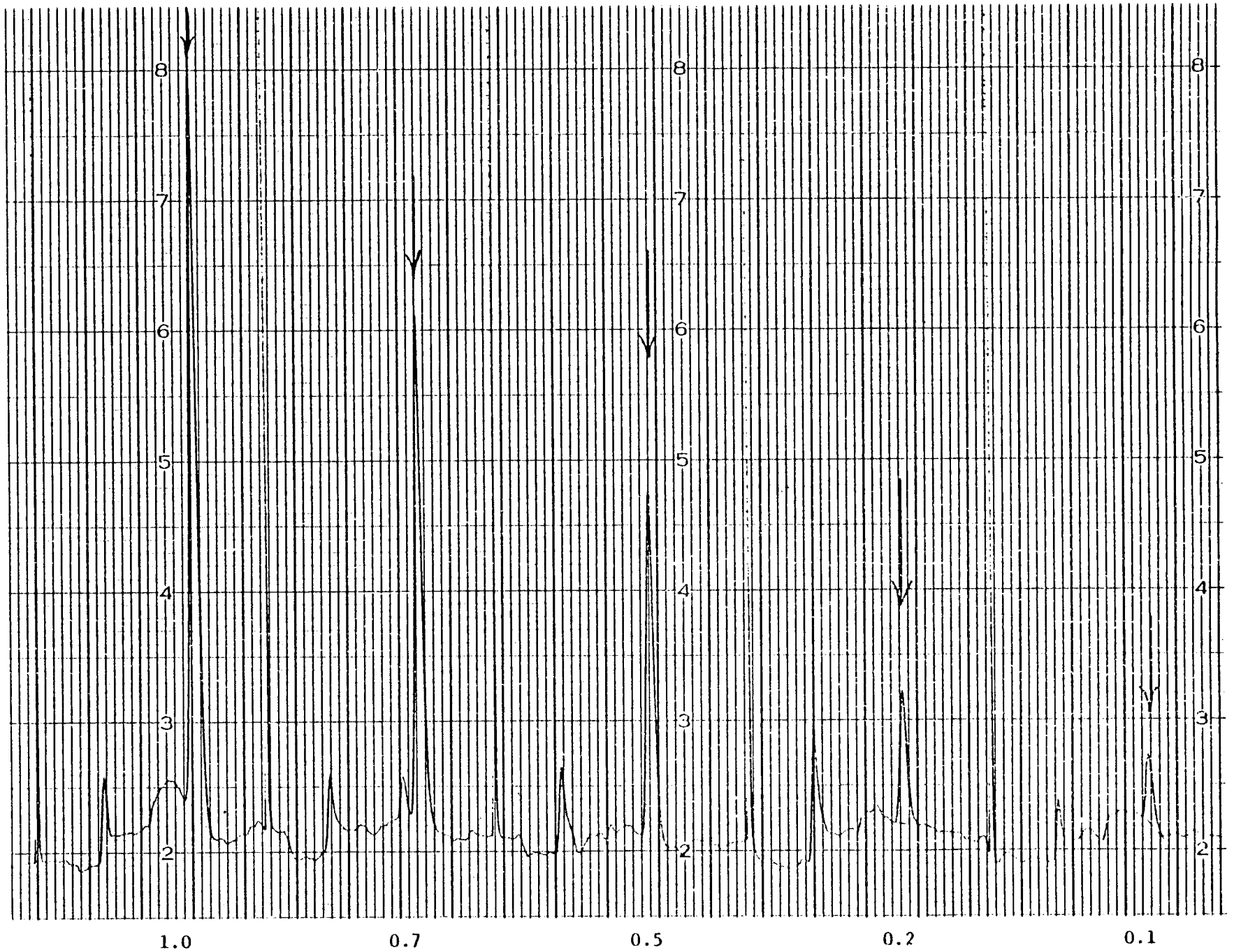


Figure 5

Typical Chromatograms of Sulfanilamide Acetyl Derivative



GRAPH 2

ACETYLSULFANILAMIDE

CALIBRATION CURVE

Automated Operation

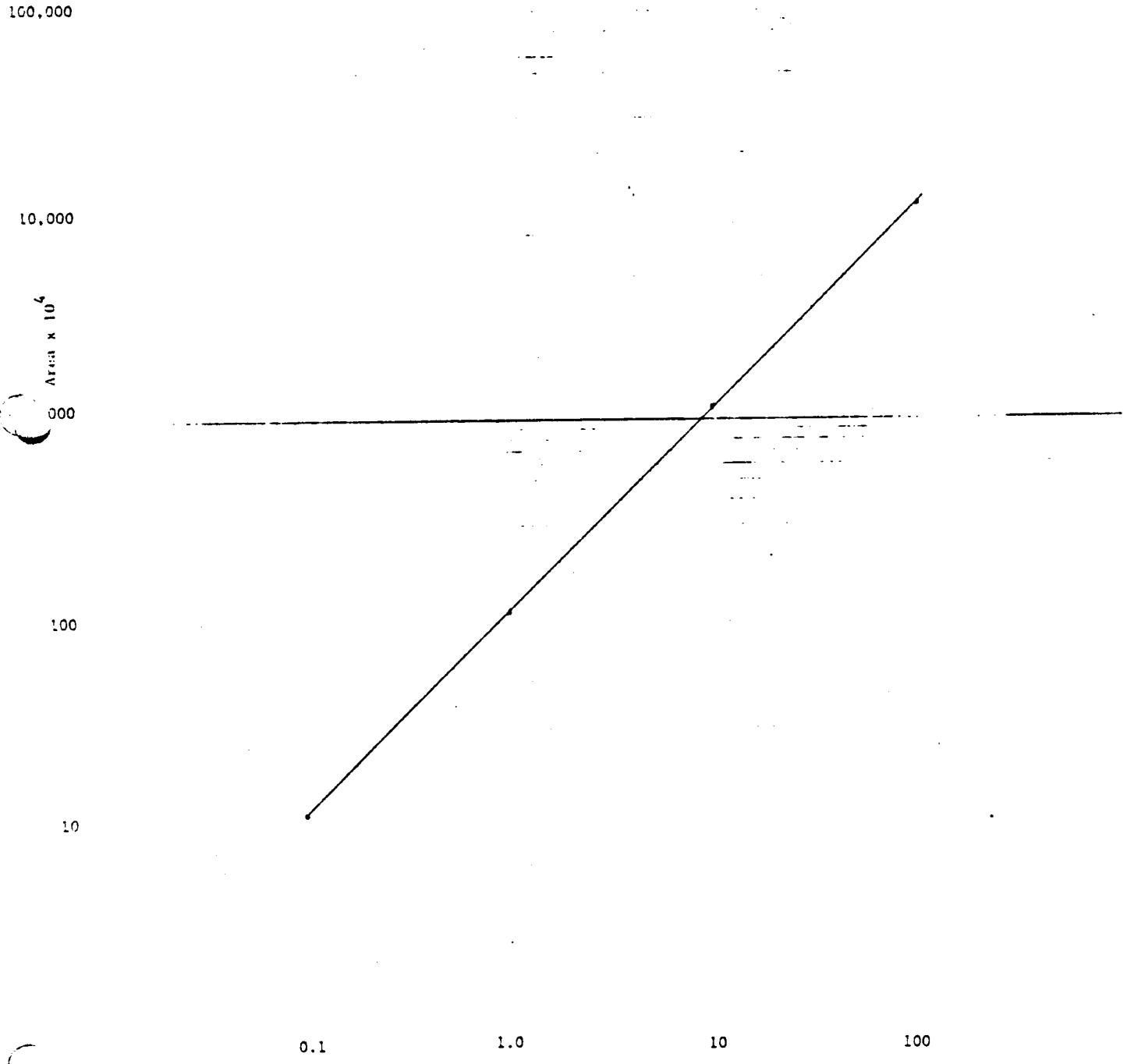


FIGURE 6

TYPICAL CHROMATOGRAMS OF THE ACETYL DERIVATIVE
AUTOMATIC OPERATION

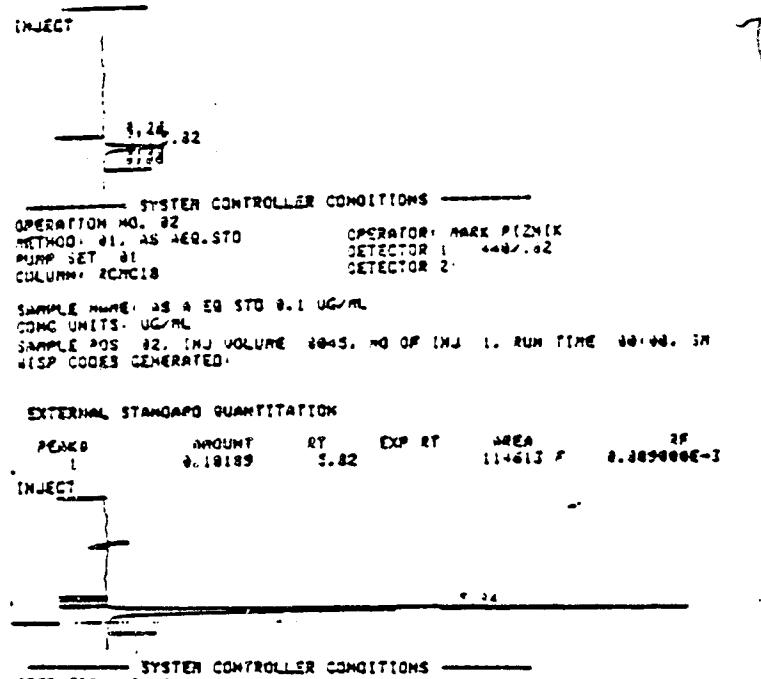
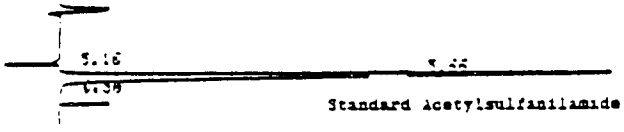


FIGURE 7

HPLC Charts of Some Sulfa Drugs

Processed Through the Procedure

INJECT



Standard Acetylsulfanilamide

SYSTEM CONTROLLER CONDITIONS

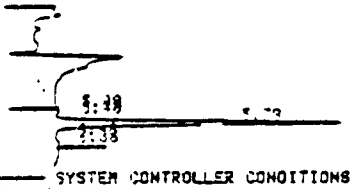
OPERATION NO. 13
METHOD: 01, TOTASU.185 OPERATOR: MARK PIZNIK
PUMP SET: 01 DETECTOR 1: 440/.02
COLUMN: RCM C18 DETECTOR 2:

SAMPLE NAME: WS A EQ STD 1 UG/ML
CONC UNITS: PPM

PEAK#	AMOUNT	RT	EXP RT	AREA	RF
1	0.50000	5.66		1153039 F	0.433600E-3

PEAK#	RT	AMOUNT	RF
1	5.67	0.50000	0.439000E-3

INJECT



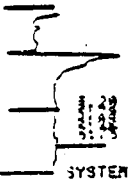
SYSTEM CONTROLLER CONDITIONS

OPERATION NO. 02
METHOD: 01, TOTASU.185 OPERATOR: MARK PIZNIK
PUMP SET: 01 DETECTOR 1: 440/.02
COLUMN: RCM C18 DETECTOR 2:

SAMPLE NAME: 500 STP SUG/5ML Sulfaquinoxaline
CONC UNITS: PPM
SAMPLE POS 02, INJ VOLUME 0045, NO OF INJ 1, RUN TIME 00:08, 3M
MISP CODES GENERATED:
EXTERNAL STANDARD QUANTITATION

PEAK#	AMOUNT	RT	EXP RT	AREA	RF
1	0.24221	5.78		549487 F	0.440800E-3
TOTAL	0.24221				

INJECT



SYSTEM CONTROLLER CONDITIONS

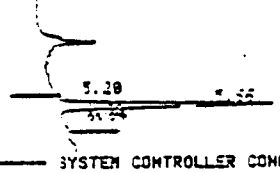
OPERATION NO. 02
METHOD: 01, TOTASU.185 OPERATOR: MARK PIZNIK
PUMP SET: 01 DETECTOR 1: 440/.02
COLUMN: RCM C18 DETECTOR 2:

SAMPLE NAME: STA STP SUG/5ML Sulfathiazole
CONC UNITS: PPM

SAMPLE POS 03, INJ VOLUME 0045, NO OF INJ 1, RUN TIME 00:08, 3M
MISP CODES GENERATED:

NO PEAKS, TRY AGAIN!

INJECT



SYSTEM CONTROLLER CONDITIONS

OPERATION NO. 14
METHOD: 01, TOTASU.185 OPERATOR: MARK PIZNIK
PUMP SET: 01 DETECTOR 1: 440/.02
COLUMN: RCM C18 DETECTOR 2

SAMPLE NAME: INT STUDY SMO W/AL COL 5/5 Sulfadimethoxine
CONC UNITS: PPM
SAMPLE POS 25, INJ VOLUME 0045, NO OF INJ 1, RUN TIME 00:08, 3M
MISP CODES GENERATED:
EXTERNAL STANDARD QUANTITATION

PEAK#	AMOUNT	RT	EXP RT	AREA	RF
1	0.24423	5.66		466663 F	0.439800E-3
TOTAL	0.24423				

APPENDIX II

YIELD DATA ON FORMATION OF N^4-ACETYLSULFANILAMIDE FROM ASULAM, ACETYLASULAM AND SULFANILAMIDE VIA ACETYLATION AND HYDROLYSIS STEPS (without biological substrates)

Acetylation and Hydrolysis Conditions:

As directed in Method No. 154

Chemical	Amount µg	Yield %
Asulam	100	88.8
	100	87.5
	100	86.1
	50	89.9
	10	89.9
	7	84.5
	5	95.7
	2	85.6
Sulfanilamide	100	73.0
	100	80.0
	100	76.9
	100	76.9
	10	84.0
	5	76.2
	2	81.6
Acetylasulam	2	83.6[a]
Asulam	1	85.7
Sulfanilamide	1	
Acetylasulam	0.2	90.5[a]
Asulam	0.1	90.5
Sulfanilamide	0.1	
Over-all Yield		84.6

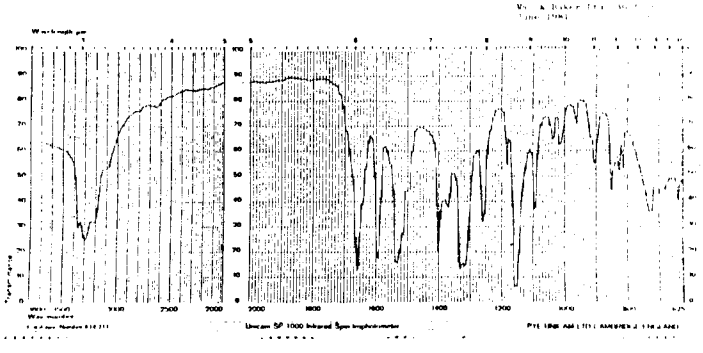
a) Duplicates of combination standards

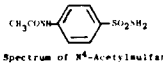
PHOTOGRAPH No. 1

Infrared (IR) Spectrum

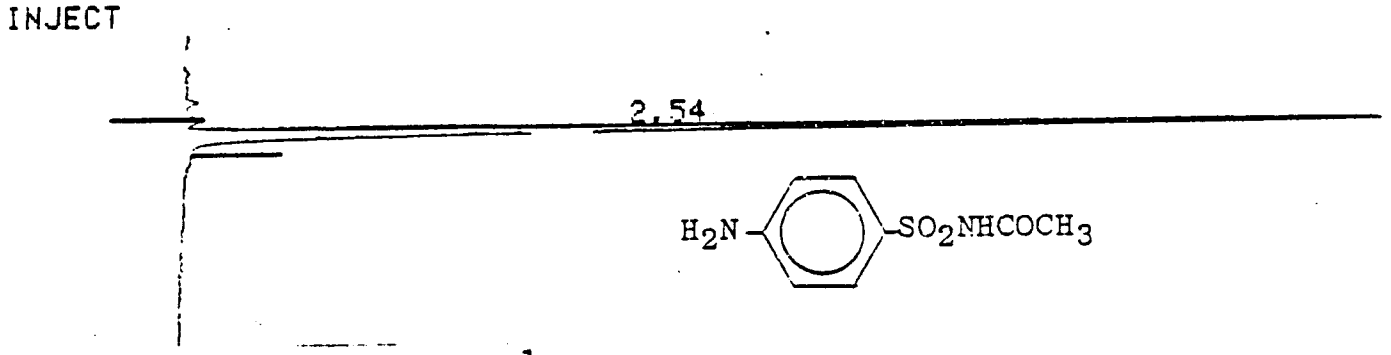
of

N^4-Acetylsulfanilamide

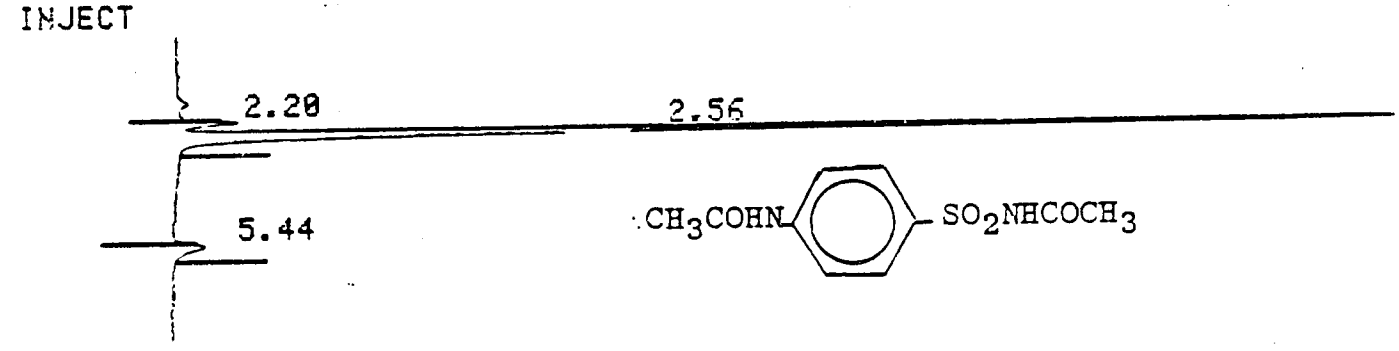




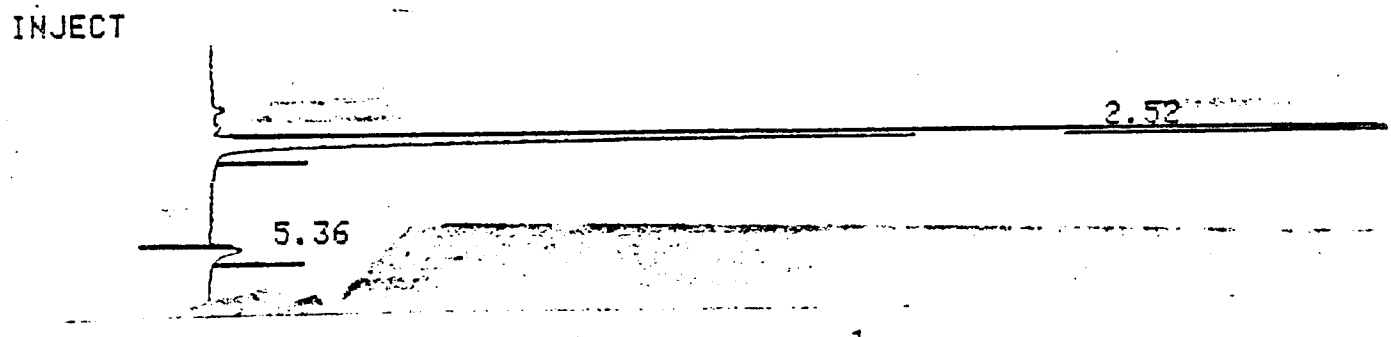
Infrared Spectrum of N^4-Acetylsulfanilamide



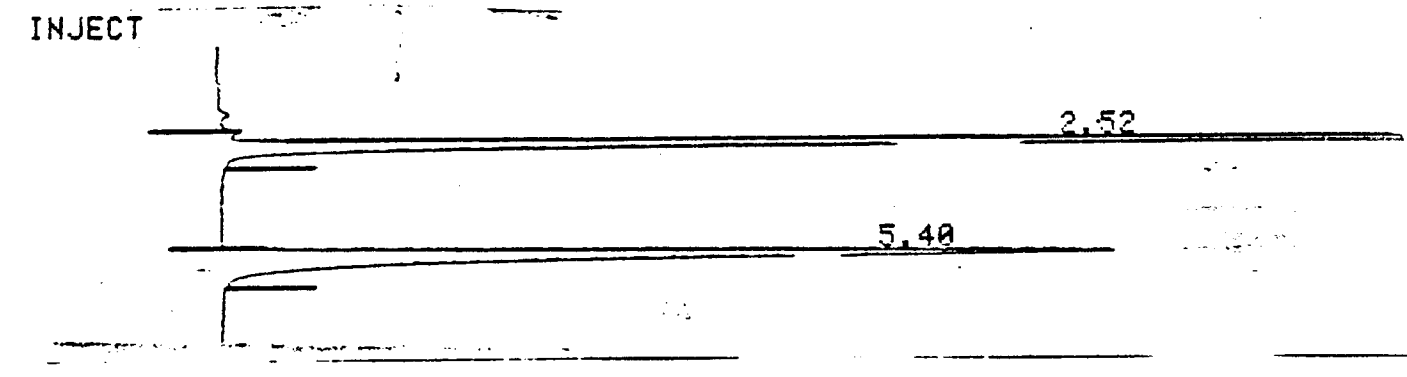
1 µg/ml N^1-Acetylsulfanilamide



1 µg/ml N^1, N^4-Diacetylsulfanilamide



Combination 1 µg/ml EACH N^1-AS
N^1, N^4-DAS



Combination 1 µg/ml EACH N^1-AS
N^1, N^4-DAS
N^4-AS

HPLC of Acetyl Derivatives of Sulfanilamide

Under the operating conditions described in Rhone-Poulenc Analytical Method No. 154, the compound of interest N^4-acetylsulfanilamide (N^4-AS) emerges at the retention time of 5.4. Other acetylated sulfanilamides, N^1-acetylsulfanilamide; N^1, N^4-diacetylsulfanilamide (N^1-AS; N^1, N^4-DAS) under the same conditions, superimpose at the retention time of about 2.5, as shown in the chromatogram enclosed.

Source of Standards:

N^1-AS - ICN - K&K Laboratories, Inc., Plainview, N.Y.
N^1, N^4-DAS - Aldrich Chemical Co., Inc., Milwaukee, WI.
N^4-AS - May & Baker, Ltd.