I. SUMMARY AND INTRODUCTION

A. SCOPE

This method is to be used for the semi-quantitative determination of Triasulfuron, N-(6-methoxy-4-methyl-1,3,5-triazin-2-yl-aminocarbonyl)-2-(2-chloroethoxy) benzenesulfonamide, CAS# 82097-50-5, in water and soil. The limit of determination (LD, the smallest dose that yields a response that is statistically significant different than the response of the zero dose) is 0.03 ppb of triasulfuron in water and 0.10 ppb in soil. The limit of detection for the method (LS, the mass of test substance contained in the Limit of Determination) is 5 and 10 picograms (pg) in water and soil, respectively.

B. PRINCIPLE

Water samples do not require preparation prior to analysis other than measuring sample pH. A soil sample (2.0 g) is extracted by vortexing and sonication in 12 ml of 25% MeOH/phosphate buffer (0.07M phosphate buffer, pH 7.1). The resulting suspension is centrifuged. Six-ml of the supernatant is combined with 9-ml of water and acidified with phosphoric acid. This solution is passed through a C-8 Bond Elut solid phase extraction cartridge. Triasulfuron is eluted with $MeCl_2$ and passed through a column of Na_2SO_4. The eluate is then blown to dryness under a stream of nitrogen. Residue is reconstituted in 1-ml of TRIS-HCl buffer (0.01M, pH 7.2).

A 100-µl aliquot of a water sample or soil extract is added to a microtiter plate coated with anti-Triasulfuron antibody. The assay is carried out by sequential addition of enzyme conjugate, wash solution and color reagent. The reaction is terminated by acidification.

Quantitation is performed spectrophotometrically at 450 nm.

A flow diagram for the analysis of water samples is presented in Figure 1. Figure 2 displays a flow diagram for the analysis of soil samples.

II. MATERIALS AND METHODS

A. APPARATUS

1.0 Experimental EnviroGard Triasulfuron Plate kit, immunoassay microplate assay, Millipore Corporation, Bedford, MA 01730.

2.0 Microtiter plate reader, Multiskan MCC/340 MK II, ICN catalog #78-626-00, or equivalent.

3.0 AUTOmate™ microplate software and manual, ICN cat. #78-599-02 and 78-599-98, respectively, or equivalent.

4.0 Microtiter plate shaker, Lab-Line Instruments catalog #4625 or equivalent.

5.0 Eight-channel pipette capable of dispensing 50- and 100-µl volumes, Elkay Labsystems catalog #4142-417 or equivalent.

6.0 Adjustable microliter pipette, capable of dispensing 20- to 200-µl volumes, Rainin cat. #P-200 or equivalent.

7.0 Adjustable microliter pipette, capable of dispensing 500- to 5000-µl volumes, Rainin cat. #P-5000 or equivalent.

8.0 Pipette tips for eight-channel pipette for volumes of 20- to 200-µl, packaged in an eight by

twelve array, Costar cat. #4865 or equivalent.

9.0 Pipette tips for single-channel pipette for volumes of 100- to 1000-µl, packaged as 100 tips per rack, Costar cat. #4867 or equivalent.

10.0 Pipette tips for single-channel pipette for volumes of 500- to 5000-µl, packaged bulk 1000 tips with 100 filters Rainin cat. #C-5000 or equivalent.

11.0 Graduated pipette, 2-, 4-, 6- and 10-ml, Fisher Cat. #13-660B,D,F and 13-650G or equivalent.

12.0 Microtiter assay plate, 96 well, flat bottom, polystyrene, non-sterile, not treated for cell culture work, Flow Laboratories cat. #76-102-05 or equivalent.

13.0 Reagent reservoirs for multichannel pipettes, non-sterile, ICN Cat. #77-824-01 or equivalent.

14.0 pH meter, Corning M-90 pH Stick, VWR cat. #34102-081 or equivalent.

15.0 Magnetic stir bar, 0.5-1.0 inch in length, Fisher cat. #14-511-61 or 14-511-63 or equivalent.

16.0 Magnetic stirrer, Fisher Cat. #11-495-28 or equivalent.

17.0 Nalgene Oak Ridge polyallomer centrifuge tube, 30 ml capacity, with polypropylene screw caps, Fisher cat. #05-529-1C or equivalent. Tubes should be capable of withstanding approximately 20,000 x G in a refrigerated centrifuge and be resistant to ethyl acetate and methanol.

18.0 Concentration tube, 15 ml capacity, Fisher cat. #05-490 or equivalent.

19.0 Vortex-Genie 2 mixer, Fisher cat. #12-812 or equivalent.

20.0 Large sample set accessory to Vortex Genie 2 mixer, Fisher cat. #12-812-2 or equivalent.

21.0 Ultrasonic cleaning bath, Branson B5200R1, Baxter cat. #C6445-52 or equivalent.

22.0 Sorvall RC-5B refrigerated centrifuge, Sorvall Instruments, Wilmington, DE 19898 or equivalent.

23.0 Eight position rotor for Sorvall RC-5B refrigerated centrifuge, 30 mm tube diameter capacity, model SS-34, Sorvall Instruments, Wilmington, DE, 19898 or equivalent.

24.0 Nitrogen evaporator, Organomation N-EVAP analytical evaporator, model #115 or equivalent.

25.0 Solid phase extraction cartridge, Analytichem Bond Elut, C-8 bonded phase (500 mg) bed, 2.8 ml column volume, Varian part #1210-2029.

26.0 Solid phase extraction vacuum manifold, Supelco cat. #5-7030M or equivalent.

27.0 Luer stopcocks for vacuum manifold, Alltech cat. #211524 or equivalent.

28.0 Solid phase extraction reservoir, empty, without frits, 15 ml capacity, Varian cat. #AL-121310-16 or equivalent.

29.0 Adaptors to couple solid phase extraction reservoirs to solid

phase extraction cartridges, Varian cat. #AL-121310-01 or equivalent.

30.0 Poly-prep column, BioRad cat. #731-1550 or equivalent.

31.0 Poly-prep stack cap, BioRad cat. #731-1555 or equivalent. Puncture opening in cap before use.

32.0 Porcelain mortar and pestle, 70 mm O.D. Fisher cat. #12-961AA or equivalent.

33.0 Stainless steel sieve, USA Standard Testing Sieve, No. 6 mesh, Fisher cat. #04-881-10D or equivalent.

34.0 Stainless steel wire whisk, approximately 30 cm in length, Cole-Parmer cat. #G-07302020 or equivalent.

35.0 Stainless steel mixing bowl, heavy gauge, approximately 32 cm in diameter, Cole-Parmer cat. #G-07300-80 or equivalent.

36.0 Parafilm laboratory sealing film, Fisher Cat. #13-374-5 or equivalent.

37.0 Disposable borosilicate culture tube, 12 x 75 mm, with white label area, Fisher cat. #14-957-22A or equivalent.

B. REAGENTS

1.0 Distilled, deionized water (H_2O).

2.0 TRIZMA BASE (TRIS), Reagent Grade, Sigma cat. #T-1503 or equivalent.

3.0 Hydrochloric acid (HCl), Reagent ACS, Fisher cat. #A144-212 or equivalent.

4.0 0.01M TRIS-HCl buffer, pH 7.2 - In 1 liter of H_2O dissolve 1.21 g TRIS and add concentrated HCl to obtain a pH of 7.2.

5.0 Acetonitrile (ACN), HPLC grade, Fisher cat. #A988-4 or equivalent.

6.0 Methylene chloride ($MeCl_2$), HPLC grade, Fisher cat. #D143-4 or equivalent.

7.0 Methanol (MeOH), HPLC Grade, Fisher cat. #A452-4 or equivalent.

8.0 Ethyl Acetate (EtOAc), HPLC Grade, Fisher cat. #E195-4 or equivalent.

9.0 Phosphoric Acid 85%, certified ACS, Fisher cat. #A242-500 or equivalent.

* 10.0 Sodium Phosphate monobasic monohydrate, (NaH_2PO_4) Fisher cat. #S369-500 or equivalent.

* 11.0 Sodium Hydroxide solution, 50% w/w, Fisher cat. #SS254-4 or equivalent.

12.0 0.07 M Phosphate Buffer, pH 7.1 - In 1 liter of H_2O, dissolve 9.66 g NaH_2PO_4 and adjust to pH 7.1 with 50% NaOH.

13.0 Soil extraction solvent - 25% MeOH/0.07 M Phosphate buffer, pH 7.1.

14.0 Acidified H_2O - To 10 ml H_2O, add 200µl concentrated phosphoric acid to achieve a pH < 2.0.

15.0 Bond-Elut prep-solution - In a 1L flask, combine 300 ml H_2O, 200 ml soil extraction solvent and 10 ml concentrated phosphoric acid.

16.0 Sodium sulfate (Na_2SO_4), anhydrous, A.C.S. certified, Fisher cat. #S421-3 or equivalent.

17.0 Sodium sulfate drying column - Add approximately 3.5 g sodium sulfate to a poly-prep column or fill to a depth of approximately 4 cm. Attach a poly-prep stack cap.

18.0 Enzyme conjugate, included in the experimental Triasulfuron Plate kit, Immunosystems Inc.

19.0 Chromogen solution, included in the experimental Triasulfuron Plate kit, Immunosystems Inc.

20.0 Substrate solution, included in the experimental Triasulfuron Plate kit, Immunosystems Inc.

21.0 Color reagent, prepared by combining two volumes of substrate solution and one volume of chromogen solution. This solution is not stable and should be used within thirty minutes of preparation.

NOTE: A single component color reagent may be substituted by the manufacturer in place of the two component system described here. Equivalent results can be obtained using either reagent system.

22.0 Sulfuric acid (concentrated H_2SO_4), Reagent A.C.S., Fisher cat. #A300S-212 or equivalent.

23.0 Stop solution (2.0 N H_2SO_4), prepared by adding 28-ml of concentrated H_2SO_4 to 472-ml of H_2O.

24.0 Triasulfuron analytical standard, Ciba-Geigy Corporation, P. O. Box 18300, Greensboro, NC 27419-8300. Storage conditions: Frozen

25.0 Refer to Table V for a listing of the remaining test substances, their characteristics, storage conditions, source and supplier address. Refer to Figure 5 for the chemical structures of all test substances.

C. ANALYTICAL PROCEDURE

The antibody-coated plate, all reagents and standard solutions must be warmed to room temperature, approximately 22°C, prior to use. Reaction kinetics are directly proportional to temperature. Be certain all solutions are warmed to room temperature before running the assay.

1.0 Sample Preparation

The preparation of water samples is described in section 1.1. For preparation of soil samples, refer to 1.2.

1.1 Preparation of Water Samples

1.1.1 Water samples are received and stored refrigerated until use.

1.1.2 The pH of each sample should be measured prior to assay. To do this, warm each sample to room temperature. Set the sample container on a magnetic stirrer and add a small stir bar. Allow the sample to stir for a few seconds prior to inserting the electrode. Record the value obtained. Samples having a pH value between 5.5 and 9.0 may be analyzed by

this method. Samples having a pH outside this range are unsuitable for this assay.

1.2 Preparation of Soil Samples

1.2.1 Soil samples are received and stored frozen until use. Samples may be thawed overnight under refrigeration or at room temperature immediately prior to use.

1.2.2 Hand sift the entire sample with a stainless steel sieve into a stainless steel mixing bowl; discard roots, twigs, rocks or other debris held back by the sieve. Manually mix the contents of the bowl with the stainless steel wire whisk for about one minute.

NOTE: If the soil sample is clumped and will not pass through the sieve, grind the sample into finer pieces with a mortar and pestle.

1.2.3 The moisture content of each soil sample must be determined to correct for the amount of soil actually analyzed (refer to Section II.J). Moisture determination may be carried out gravimetrically by

weighing an aliquot before and after drying overnight in an oven set to approximately 100°C. This determination may be done prior to or coincident with immunoassay analysis.

2.0 Sample Extraction (Soil)

2.1 Weigh 2.0 g of soil into a 25 ml centrifuge tube. Sample fortification, if required, should be done at this time.

NOTE: Refer to Section II.F.1.0 for instructions on how to fortify soil samples.

2.2 Add 12 ml of the soil extraction solvent. Cap the centrifuge tube and place the tube into the foam insert fixed to the top of the Vortex Genie 2. Be sure one of the large rubber bands encircles the tube. Set the sample to vortex at the highest speed setting for 10 minutes.

2.3 Remove the tube from the foam insert and place the tube in the sonicator. Sonicate for 10 minutes.

2.4 Transfer the tube to the centrifuge and spin down the tube's contents at about 4°C at approximately 12,000 RPM for 20 minutes. The centrifuge tube cap should be loosened before centrifugation to prevent the cap from freezing tight. If a glass tube is used, an adaptor sleeve should be fitted to the tube to guard against tube breakage. When

the sample is removed from the centrifuge, take care not to disturb the pellet.

3.0 C-8 SPE Cleanup

3.1 Attach a C-8 Bond Elut cartridge to a Luer stopcock and then to the solid phase extraction manifold. Add an adaptor to the cartridge and attach a 25 ml reservoir. Pre-condition the cartridge by sequential addition of 5-ml MeOH and 5-ml column prep. solution.

3.2 Add 6 ml of the supernatant to the reservoir followed by 9-ml H_2O and 250 µl of concentrated phosphoric acid. Pass the solution through the cartridge under low vacuum (approx. 3-5 inches of Hg). Do not allow the column to go dry.

3.3 Wash the cartridge by addition of 2 ml acidified water followed by 10 ml water using low vacuum as per 2.6. After all the water has been added to the cartridge, draw air through the packing for 10 minutes. Blow out any remaining trapped water using a pipette bulb. It is important that the manifold tubes are dry. Rinse all manifold tubes with MeOH and dry tubes under nitrogen before proceeding to Step 2.8.

3.4 Prepare the manifold system for eluting the analyte from the cartridge. Fit each manifold tube with a concentration tube to collect the cartridge eluate. Remove the cartridge from the manifold and the

stopcock. Attach the sodium sulfate drying column fitted with a stack/cap to the base of the cartridge. Add a clean, unused stopcock to the base of the drying column and attach the set-up to the manifold.

3.5 Elute the analyte by adding 4 ml of $MeCl_2$ to the cartridge reservoir. Apply low vacuum pressure as per step 2.6. After all the $MeCl_2$ is collected, transfer the concentration tube to a warm water bath (approximately 37°C) and reduce the solution to dryness under a gentle stream of nitrogen.

NOTE: Completion of step ~~2.9~~ 3.5 can serve as a break point in the method. After the solutions in all tubes have been reduced to dryness, the tubes may be sealed with parafilm and stored under refrigerated conditions overnight before proceeding to the next step. Be certain to allow the tubes to come to room temperature before Section II.C.3.6.

3.6 Add 1 ml 0.01 M TRIS-HCl to the tube. Sonicate this solution for 10 minutes and vortex briefly.

NOTE: The opening a concentration tube may be too narrow to use a 200 µl pipettor to transfer 150 µl aliquots to the reservoir plate in Section II.C.4.1.1, below. Should this occur, transfer the solutions to 12 x 75 mm culture tubes. Be certain to label the culture tubes properly

prior to transfer to avoid misplacing sample solutions.

4.0 Enzyme Immunoassay

4.1 Inhibition of Enzyme Conjugate

4.1.1 Approximately 150 µl of each water sample, TRIS-HCl soil extract or standard solution is added individually to wells of the uncoated reservoir plate as indicated on the plate layout chart previously completed by the analyst. A sample layout chart is illustrated in Figure 3.

4.1.2 Adjust an eight-channel pipette to deliver 100 µl (refer to the pipette operation manual for operation of the multichannel pipettes). Snugly fit eight tips to the pipette and transfer a 100-µl aliquot from column 1, rows A through H, of the reservoir plate to the corresponding column on the antibody-coated assay plate. Eject the used tips to a waste receptacle. Attach eight new tips and proceed to transfer aliquots from columns 2 through 12 of the reservoir plate to the assay plate taking care to change

tips after each transfer. If an alternative, equivalent pipetting device is used in lieu of a hand-held multi-channel pipette, the 100-µl aliquot from each well of the reservoir plate will be transferred to the assay plate in a manner consistent with the operation of the device.

4.1.3 Decant the enzyme conjugate solution from its container into a multichannel pipette reagent reservoir.

4.1.4 As soon as all sample and standard solutions have been transferred to the assay plate, successively pipette 100-µl aliquots of the enzyme conjugate solution from the reagent reservoir to each column of the assay plate from left to right as described in Section II.C.4.1.3. The pipette tips must be changed after each transfer.

Note: The enzyme conjugate solution has a surfactant-like character and tends to bubble and foam when pipetted. Through preliminary trials the analyst should ensure that manual or

automated pipetting devices reproducibly deliver 100-µl aliquots without bubbling or foaming.

4.1.5 Place the plate on the plate shaker. Start the shaker with the power control set to the "constant" position. The speed setting should be adjusted to approximately ninety oscillations per minute or slightly less than "2" on the Lab Line shaker. Allow the plate to shake for one hour.

Note: The reaction kinetics of this assay are proportional to temperature. To avoid exposing the plate to harsh fluctuations in temperature such as strong drafts, the plate shaker should be placed inside an opaque closed chamber. Placing a cardboard box over the shaker will suffice if a more elaborate apparatus, such as a plexiglas chamber, is not available.

4.2 Wash

Remove the plate from the shaker. Using a hand-held multichannel pipette, remove the reactants from each column left to right across the plate. Starting with column

one, add about 200-µl of H_2O to each well, gently agitate the plate and immediately remove the wash solution. Proceed across the plate from column two to twelve in a similar fashion.

Wash the plate two additional times. After the final wash, shake the plate vigorously to remove most of the remaining liquid. The plate may be inverted and blotted with dry paper towels. Blot only the exterior of the plate. Do not insert the blotting paper into the wells. Visually examine the plate to ensure little wash remains.

Should only part of the microtiter plate be used, the analyst should exercise care not to contaminate unused wells.

If an alternative washing device is used, be certain to remove the reactants in a manner consistent to their addition.

4.3 Color Development

4.3.1 Prepare fresh color reagent as described in Section II.B.5. II.B. 21 This solution is unstable and should be made up not longer than five minutes before Section II.C.4.3.2 is performed. Add 150-µl of color reagent to each well across the plate in the same fashion as the previous solutions

were dispensed. Tips need not be changed between additions.

4.3.2 Place the plate on the plate shaker and shake for approximately 0.5 hour with the controls set at the same settings as described in Section II.C.4.1.5. Individual wells will gradually turn varying shades of blue. The plate may be left to develop up to one hour at the discretion of the analyst if more intense color development is desired.

4.3.3 When the analyst chooses to terminate color development, stop the reaction by adding 50-µl of stop reagent to each well in the same fashion as the color reagent was added. Be sure to change tips between each addition to avoid contaminating a column of wells with residue from a previous column. Mix the acidified solution well by repeated pipetting and dispensing of each well's contents, taking care not to form bubbles or to leave liquid behind in the pipette tips. Should bubbles form, they can be broken by manipulation with a pipette tip. Tapping the side of the plate

gently may aid mixing of acidified solution. A well mixed solution will appear yellow to the eye with no traces of blue remaining. The plate should be analyzed within 30 minutes of addition of stop reagent.

D. INSTRUMENTATION

1.0 Description and Operating Conditions

1.1 The Titertek Multiskan MCC/340 MK II eight-channel filter spectrophotometer is used to measure the absorbence of the final reaction solutions in each well. The operator should select filter 4 (450 nm) for this method. The instrument should be warmed up for ten minutes prior to use.

Refer to the instrument operating instructions for further details on the operation of this instrument.

2.0 Standardization

2.1 Each assay consists of standards and samples run concurrently on the same plate. The absorbance values obtained from sample solutions may only be compared to the absorbance of standards run on the same plate. Triasulfuron standards range from 2.0 to 0.05 ng/ml in addition to a zero dose. Standards for soil and water samples are dissolved in H_2O.

2.2 Spectrophotometric analysis of the colored reaction products obtained from standard solutions will yield absorbance values measured at 450 nm (A_{450}). With a calculator or computer, use these data to generate a log/linear regression function. This curve is in the form of y = m log x + b. The concentration of the Triasulfuron standards are plotted on a logarithmic scale on the horizontal (x) axis and the absorbance values are plotted on a linear scale on the vertical (y) axis. An example of a typical standard curve is shown in Figure 4.

E. INTERFERENCES

1.0 Refer to Section III.C. SPECIFICITY for a detailed discussion of the cross-reactivity parameters of this assay.

2.0 No interferences were observed in control soil or water samples.

F. CONFIRMATORY TECHNIQUES

1.0 The ability of the microplate assay to respond to triasulfuron in soil and water may be assessed by the analysis of samples fortified with a known amount of Triasulfuron. Five ml of a water sample may be spiked with 50-µl of a 100-, 10- or 5-ng/ml standard solution to yield a fortified sample with a final concentration of, respectively, about 1.0, 0.10 or 0.05 ppb greater than that of the original unfortified sample. Soil (2.0 g) may be fortified by addition of 100 µl of a 200-, 20- or 2-ng/ml standard solution to yield a 10, 1.0 or 0.1 ppb fortification,

respectively. Refer to section II.I.2.0 for the preparation of fortification standards for soil and water samples.

2.0 Triasulfuron residues greater than or equal to 0.1 ppb in water determined by this method can be confirmed by high performance liquid chromatography (HPLC) according to Minnesota Valley Testing Lab method MVTL T75123 (1). Residues greater than or equal to 1.0 ppb in soil determined by this method can be confirmed by high performance liquid chromatography-mass spectrometry (LC/MS) according to A.D. Little method ADL-65429-3 (2).

G. TIME REQUIRED

An analyst can analyze forty-two water samples and six standards in duplicate in approximately five hours.

Eight soil samples (including a control sample and two procedural recoveries) and six standards can be analyzed in a 24 hour period.

H. MODIFICATIONS AND POTENTIAL PROBLEMS

1.0 As previously stated in the introduction to the ANALYTICAL PROCEDURE, the microtiter plate, reagents and samples for analysis must be warmed to room temperature, approximately 22°C, before use. Placing the plate (in its plastic bag) and the reagents in a hood with the sash drawn low for approximately 30 minutes will bring the reagents to temperature efficiently.

2.0 The pH of a water sample, if extreme, may effect antibody binding. The pH of each sample should

be determined. If a sample is found to have a pH outside the range of 5.5-9.0, it is unsuitable for analysis by this method.

3.0 The bottom surface of the microtiter plate is the optical surface through which the absorbance of the final reaction product will be measured. The analyst should exercise care to prevent damage to this surface. The plate is designed to prevent the bottom from contacting flat surfaces, such as a bench top, upon which the plate may be placed. Nevertheless, the analyst should maintain a clean work area as a preventative measure.

4.0 The analyst should take care to be certain all water samples, soil extracts and standard solutions are positioned properly in the reservoir plate. The large sample load requires that special attention to detail be maintained throughout the analysis.

5.0 The length of color development time may vary as described in Section II.C.2.3.2. The absorbance at 450 nm of the blank standard should be in the range of 1.0 to 1.8 absorbance units. The absorbance will vary since the turnover rate of the enzyme conjugate generating the colored signal is dependent on ambient temperature. It is important, therefore, to bring all reagents to room temperature (as noted in 1.0 above) and avoid strong fluctuations in room temperature while the assay is in progress.

6.0 The analyst may observe A_{450} values which are less than those of the highest standard, 2.0 ng/ml. In

this event, the concentration of the corresponding sample cannot be calculated since its absorbance readings do not fall within the range of the standard curve. The sample should be diluted and reassayed to obtain absorbance readings which lie within the bounds of the standard curve. The concentration of the undiluted sample can then be calculated by multiplying the concentration of the diluted sample by the dilution factor.

7.0 The transfer of solutions by multi-channel pipettes requires the analyst to constantly monitor his or her technique. Pipetting errors are the major source of error in immunoassay methodology.

8.0 Extraction solvent should be added to soil samples immediately before vortexing.

9.0 After centrifugation, soil samples should not be allowed to sit for longer than 5-10 minutes before Bond-Elut clean-up. As the samples warm to room temperature, some re-mixing may occur.

10.0 It is important to use a low vacuum (approximately 3-5 inches of Hg) when performing the Bond-Elut isolation step. A stronger vacuum may pull off unwanted substances which could cause interference in the assay.

11.0 During evaporation of the $MeCl_2$ eluate, the analyst should use a gentle stream of nitrogen to limit the possibility of blowing the analyte out of the concentration tube. A gentle stream should cause only a slight indention on the surface of the eluate.

12.0 White crystalline residue may be observed in a concentration tube after the $MeCl_2$ eluate has been reduced to dryness. This residue does not affect the performance of the immunoassay even though addition of Tris buffer may result in a cloudy solution.

13.0 Although 4 ml of $MeCl_2$ are used to elute the analyte from the C-8 cartridge, approximately 3 ml or less is usually collected. This does not affect elution of the analyte. However, if the analyst notes that less than 1 ml is collected, the sample should be re-extracted and re-analyzed.

14.0 No problems were observed with the solubility or stability of any test substance under the conditions used in this study.

I. PREPARATION OF STANDARD SOLUTIONS

Triasulfuron standard solutions are made by dissolving 5.0 mg of Triasulfuron in 100 ml ACN and sonicating. This will make 100 ml of a 50 µg/ml solution. To make up standards for analysis of water samples, serially dilute this solution with H_2O to make 5000-, 1000-, 100-, 10-, 5.0-, 1.0-, 0.5-, 0.1- and 0.05-ng/ml standards. Also prepare a blank consisting solely of H_2O. A similar 50 µg/ml stock solution is made up in EtOAc for soil fortification standards. This stock is serially diluted in EtOAc to yield standards of 200-, 20- and 2-ng/ml. Store these standards under refrigerated conditions when not in use.

J. METHODS OF CALCULATION

Triasulfuron residues in water samples are determined by inserting the absorbance value of a given sample into the log/linear regression function generated by methods described in Section II.D.2.2.

These calculations may be made on a computer or hand-held calculator.

Triasulfuron residues found in soil samples may be corrected for the amount of moisture in each sample. If such a correction is desired, divide the residues found by the actual amount of soil analyzed. To determine the amount of soil analyzed, use the following formula:

$$\text{g sample analyzed} = \frac{g \times Ve}{Vs + (m \times g)}$$

where g is the wet weight in grams of the soil sample

Ve is the volume in ml of the aliquot transferred to the solid phase cartridge

Vs is the volume in ml of extraction solvent used

m is the percent moisture in the sample expressed as a decimal (i.e., 25% is expressed as .25).

For example, a soil sample found to contain 0.15 ppb Triasulfuron-equivalents (ppb TE) is also determined to consist of 25% moisture. Thus,

$$\text{g sample analyzed} = \frac{2 \times 6}{12 + (.25 \times 2)} = .960$$

The corrected amount of Triasulfuron found is then 0.15 ppb TE/.960 sample analyzed = 0.16 ppb TE.

After the correction for soil moisture has been accomplished, the amount of Triasulfuron residues found in soil samples may be further adjusted if the mean percent recovered of the procedural recovery samples included in that run indicates less than a quantitative extraction (100% of applied fortification recovered).

The results of all procedural recovery samples are averaged together, on a percentage basis, to obtain the mean percent recovered for the recovery

samples. If any residue is found in the control sample, the mean of the residue found (between replicate analyses of the control) is subtracted from each procedural recovery result before the mean percent recovered can be determined. If only one replicate of the control contains residue, that value is subtracted from the procedural recovery results.

Individual sample results are corrected by dividing those results by the mean percent recovered of the procedural recovery samples, if and only if, the mean percent recovered is less than 100%.

III. RESULTS AND DISCUSSION

A. VALIDATION

The objective of Protocol 356-92 was to validate "draft" AG-606 by analysis of water and soil samples also analyzed, respectively, by high performance liquid chromatography (HPLC) according to Minnesota Valley Testing Laboratory method T75123 (1) and by HPLC with mass spectrometric detection (LC/MS) by procedures described in A.D. Little method ADL-65429-3 (2).

Eighty-nine water samples were analyzed according to "draft" AG-606. The results of these analyses were compared to those obtained by HPLC. A plot of the mean of the immunoassay results versus the HPLC data (1) is shown in Figure 6. The slope of the fitted regression line is less than one (slope = 0.89) indicating the results by the HPLC are generally greater. The apparent positive bias is actually the result of two outlying points (duplicate analyses of the same sample), the two highest values on the plot (>25 ppb). When the outliers are removed (Figure 7), a more representative comparison of the two data sets is achieved. In this plot, the immunoassay values are shown to exhibit a slight positive bias (slope =

1.26) which is a typical immunochemical response.

Twenty-one soil samples from a North Dakota field dissipation study (3) were also analyzed according to procedures described in draft AG-606. A correlation of the mean of the immunoassay results and determinations obtained at A. D. Little by LC/MS is plotted in Figure 8. The results between methods correlate favorably (r = 0.88) although the slope of the fitted line is quite low (slope = 0.54). The depressed slope is due to three samples whose LC/MS values are greater than 5 ppb. Removing these high values from the comparison data set (Figure 9) results in a fitted line with increased slope (slope = 0.86) and an intercept (0.07) not significantly different than zero (P = 0.87).

It is obvious the variability between methods is greater between the soil analyses than among the results of the water experiments. The increase in variability may be due to several factors.

First, when collecting and subsampling water samples, analysts assume that an analyte is uniformly distributed throughout the sample matrix. This assumption does not hold for soil samples. As a result, the manner in which samples are collected and the actual subsample prepared for analysis becomes critical to the overall success of the analysis. These problems must be recognized when conventional chromatographic analyses are conducted but are amplified when immunochemical techniques are used due to the very small sample sizes often employed. In this study, A.D. Little personnel analyzed 20 g of each sample whereas Ciba analysts extracted 2 g per sample. Ciba personnel were thus confronted with the problem of obtaining a representative subsample of only 2 g in size. To accomplish this, prior to collecting a subsample for analysis, Ciba analysts

manually sieved the samples and then hand mixed the soils to apparent homogeneity in a stainless steel mixing bowl with a wire whisk. This strategy was successful in light of the correlation coefficients shown on Figures 8 and 9.

Second, subsamples of each sample were analyzed independently by two laboratories using different analytical techniques. That is often not the case when immunoassays are evaluated. Instead, some authors have made comparisons between techniques using a common extract (4-6). This may yield closer results between methods but often does not reflect the way each method will be put to use. Confirmatory analyses, for example, performed to verify residue detected by an immunoassay screen will probably not use the screening extract since the sample size, solvent systems and solvent volume are often very different. Extraction conditions for conventional analyses are also frequently harsher than those used for a screening technique. Consequently, employing a single extract for comparing different residue methodologies reduces the variability between methods but may yield an unrealistic comparison.

Third, soils used in this study contained incurred residues. This is in sharp contrast to the approach used by most investigators (4, 5-13). Typically, soils are freshly fortified and extracted. The extracts are subsequently analyzed by the method of choice. This technique may provide information about the suitability of a method for analysis of freshly fortified samples but not about its utility when weathered soils must be analyzed. It is striking to observe that most workers have adopted such an approach considering that nearly all samples coming in from the field will have undergone some process of weathering. Residues may have also bound to soil particles, potentially rendering facile extractions useless. Although the

results of soil analyses in this study do not correlate as closely as the water analyses, these results were produced under the same conditions as those under which the method is intended to be used. Consequently, this study presents a realistic assessment of the method's utility.

Finally, some of the disparity of results between methods may be explained by the low procedural recoveries often obtained by the LC/MS method (2). The three samples excluded from the correlation data set in Figure 9, for example, yielded much higher results by LC/MS than by immunoassay. Those results were corrected for mean procedural recoveries of only 66% (2). In contrast, immunoassay procedural recoveries averaged approximately 102% so that sample results were often unaffected by the results of the recovery samples (Table III).

All samples used in this study will be discarded at the conclusion of this project following QA verification. The raw data and final report from this study will be archived in the Biochemistry Group Archives, Room L-0003, Ciba-Geigy Corporation, 410 Swing Road, Greensboro, NC 27419.

During the course of this project, no circumstances occurred that affected the quality and integrity of the study.

C. SPECIFICITY

The specificity of this assay was determined by investigating the cross-reactivity parameters of twenty-four

structurally related test substances. These results indicate the antibodies used in this method react primarily with Triasulfuron. They also react, to a much lesser extent, with other parent sulfonylureas including CGA-152005, DPX-M6316, Metsulfuron methyl and DPX-A7881. The reactivities of these compounds relative to the reactivity of Triasulfuron (the I_{50} of Triasulfuron divided by the I_{50} of the compound of interest multiplied by 100) are 3.2, 1.0, 1.0 and <1.0 percent, respectively. Refer to Figure 5 for the chemical structures of these compounds.

D. LIMITS OF DETECTION AND DETERMINATION

The least detectable dose (limit of determination, LD) of each test substance was determined using Robard's method (14). From each LD a limit of detection (LS) was calculated. These results are summarized in Table IV. This method has a limit of detection of 3 pg of Triasulfuron in soil and water. It has a limit of determination of 0.05 ppb and 0.10 ppb in water and soil, respectively.

Although the LD of Triasulfuron has been calculated to be less than 0.05 ppb, the smallest standard is 0.05 ppb. Quantitation of sample residues will not be made lower than the smallest standard for water samples. Water samples, which give values below 0.05 ppb, will be reported as <0.05 ppb. As indicated above, soil samples may be quantitated down to 0.10 ppb.

E. LIMITATIONS

AG-606 has several limitations. First, as stated in Section II.C.1.1.2, the pH of a water sample, if extreme, may effect antibody binding. As a result, the pH of each water sample must be checked prior to analysis. If the pH of a sample is found to be outside the range of 5.5-9.0, it is unsuitable for analysis by AG-606.

Second, although the cross-reactivity data in Table I indicates the antibodies used in AG-606 bind strongly to few test substances aside from Triasulfuron, these data are not meant to be an exhaustive evaluation of the assay. The number of test substances used in this study represent a limited attempt to investigate the cross-reactivity of the assay. These results should not be extrapolated upon the potential myriad assortment of possible structurally related compounds of natural or synthetic origin. As a result, analysts should utilize this assay as a screening method. Positive results above a predetermined level of significance should be verified by a conventional, confirmatory analysis.

Third, this method is intended to complement, not replace, chromatographic analyses. It can be used as an inexpensive screening technique to remove samples that yield responses below a pre-selected level of concern from further, more expensive analytical procedures. Limited laboratory space, budgets and personnel can then be efficiently utilized to performing chromatographic analyses of samples that merit further attention.

FIGURE 1. FLOW DIAGRAM FOR AG-606 FOR THE SEMI-QUANTITATIVE DETERMINATION OF TRIASULFURON IN WATER

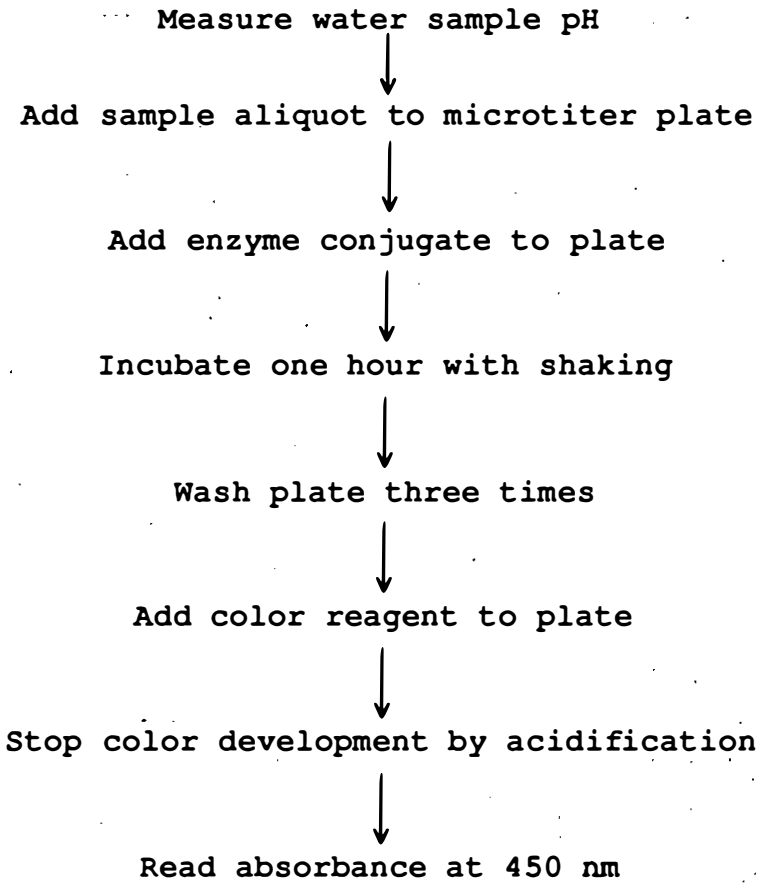


FIGURE 2. FLOW DIAGRAM FOR AG-606 FOR THE SEMI-QUANTITATIVE DETERMINATION OF TRIASULFURON IN SOIL

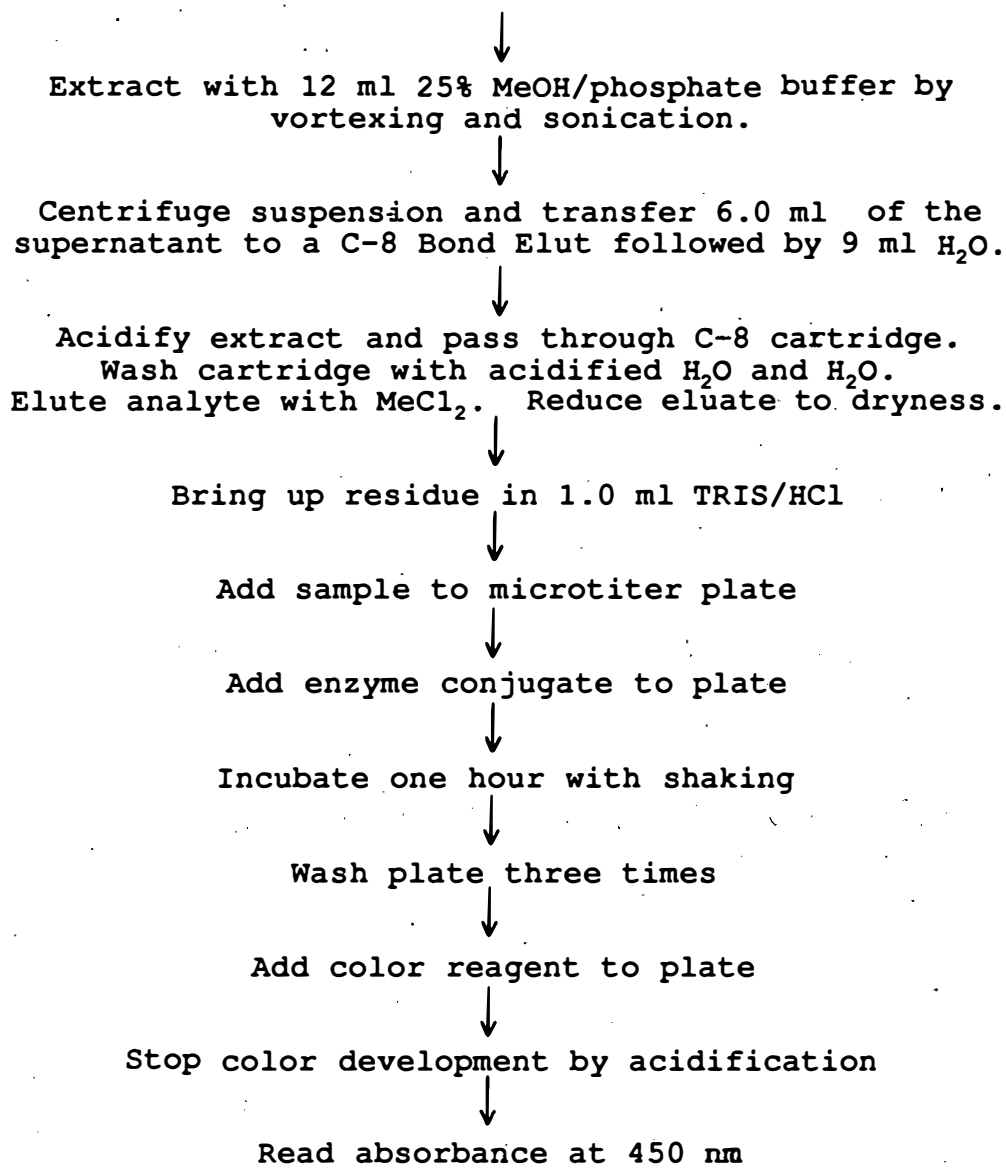
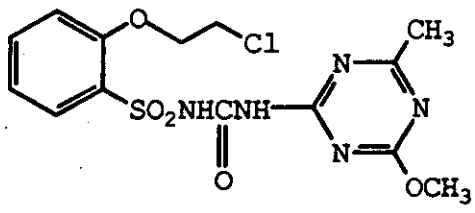
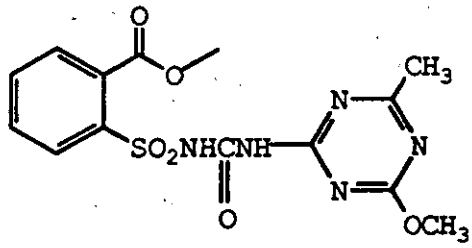


FIGURE 5. STRUCTURE OF TEST SUBSTANCES EVALUATED FOR CROSS-REACTIVITY



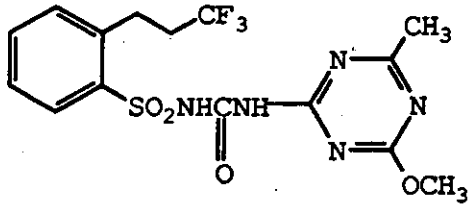
TRIASULFURON

(AMBER)

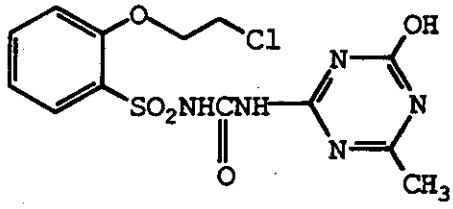


METSULFURON METHYL

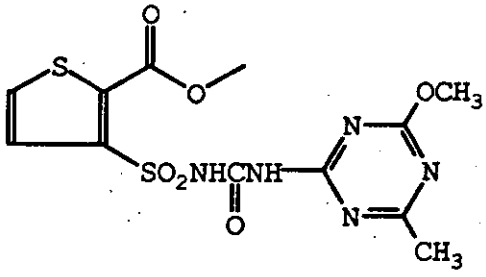
(ALLY)



CGA-152005

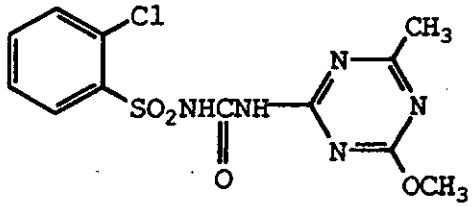


CGA-195660



THIFENSULFURON METHYL

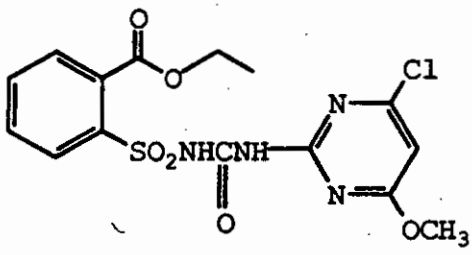
(DPX-M6316)



CHLORSULFURON

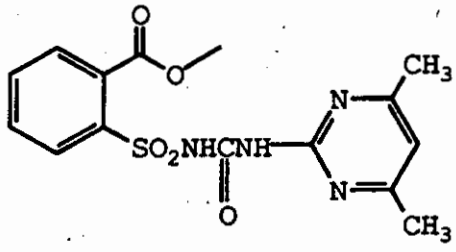
(GLEAN)

FIGURE 5. STRUCTURE OF TEST SUBSTANCES EVALUATED FOR CROSS-REACTIVITY (Continued)



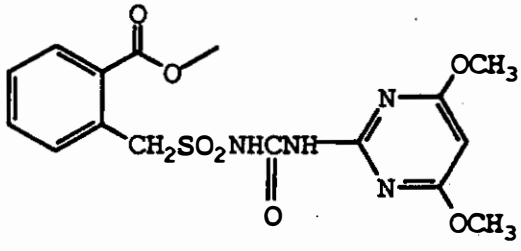
CHLORIMURON ETHYL

(CLASSIC)



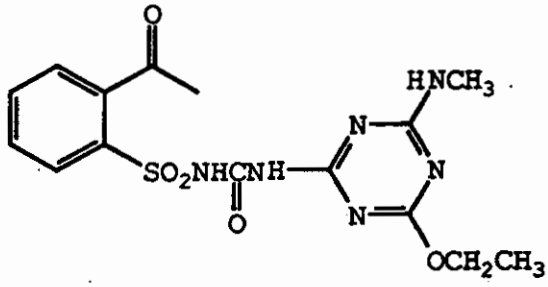
SULFOMETURON METHYL

(OUST)



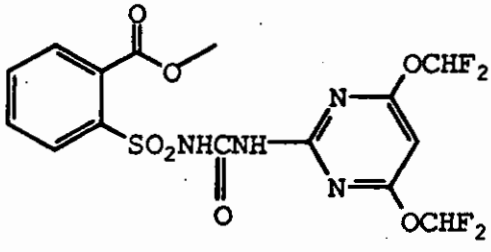
BENSULFURON METHYL

(LONDAX)



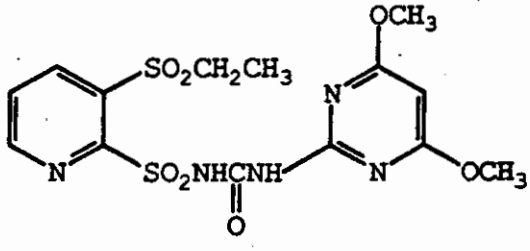
DPX-A7881

(MUSTER)



PRIMISULFURON METHYL

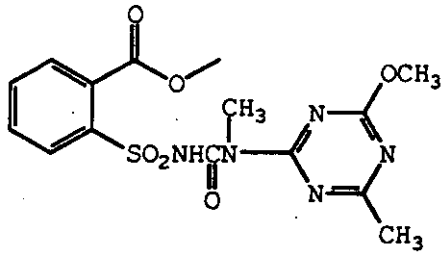
(BEACON)



DPX-E9636

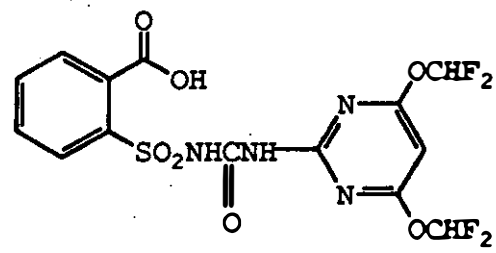
(TITUS)

FIGURE 5. STRUCTURE OF TEST SUBSTANCES EVALUATED FOR CROSS-REACTIVITY (Continued)



TRIBENURON METHYL

(EXPRESS)



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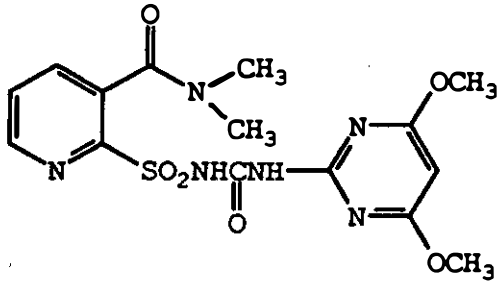
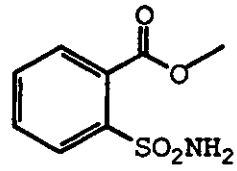


NICOSULFURON

(ACCENT)



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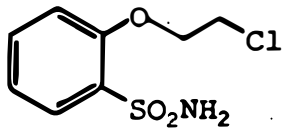

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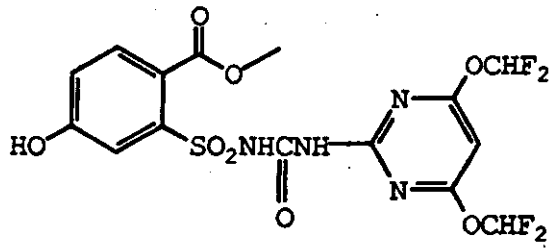
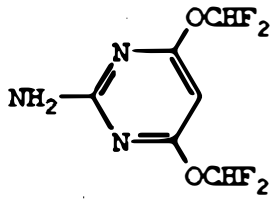
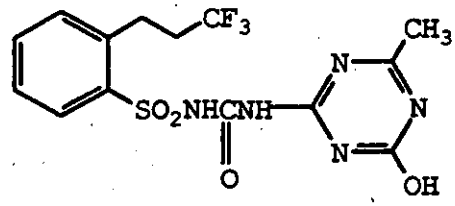


CGA-219741

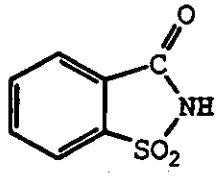
FIGURE 5. STRUCTURE OF TEST SUBSTANCES EVALUATED FOR CROSS-REACTIVITY (Continued)



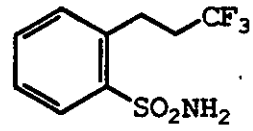
CGA-171683



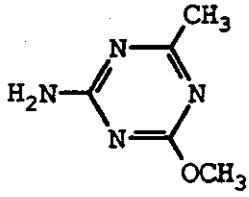
CGA-300406



CGA-27913



CGA-159902



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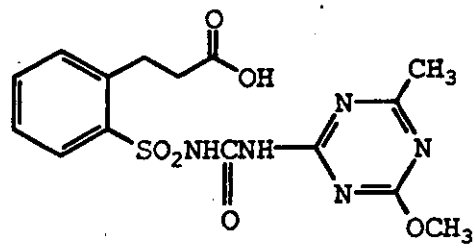

CGA-269589