SUMMARY

Triflumizole and three soil degradates, FA-1-1, FM-6-1 and FD-1-1 are extracted from soil and converted to a common moiety – namely FA-1-1 (2-amino-5-chlorobenzotrifluoride). The latter is further converted to a derivative that could be detected with increased sensitivity by GC/ECD.

A. MATERIALS

A.1 Equipment

Balances	XE-300, FX400, Fisher XL5000 and Mettler AT261
Syringe	Hamilton Microliter syringes, various volumes
Rotary Evaporator	Buchi Rotavapor™ RE 121
HPLC System	Hewlett Packard Series 1050 with Autosampler
Detector	HP Series 1050 Variable Wavelength UV/VIS
Data Base System	Dionex Chromatography System
Centrifuges	Mistral 3000E or Marathon 6 K
Vortex	Thermolyne 16700 Mixer
Nitrogen Evaporator	Organomation Analytical Evaporator
Gas Chromatograph (GC)	Hewlett Packard 5980 with ECD Detector
GC Autosampler	Hewlett Packard 7673A
GC Integrator	Hewlett Packard 3396
GC Columns	DB-225 (15 m or 30 m X 0.53 mm id X 1.0 µl film) DB-1701 (15 m X 0.32 mm id X 0.25 µl film) J & W Scientific
GC/MS	Hewlett Packard 5971A Mass Selective Detector
Desiccator	Sanplantec Corporation (Japan)
Oven	Fisher Scientific Isotemp Series 500
Distillation Apparatus	Nielsen-Kryger (Ace Glass 6556-40)

A.2 Chemicals

All solvents and reagents were obtained from Fisher Scientific, Curtin Matheson Scientific, Aldrich Chemical Company, Pierce Chemical Company, Sigma or J.T. Baker. The water used was either HPLC grade from Fisher Scientific or ASTM Type I obtained form a Barnstead NANOpure II™ system. The following solvents and reagents were utilized in the study:

Acetone
Hexane
Anhydrous Sodium Sulfate
Sodium Hydroxide
Methylene Choride
Sodium Bicarbonate

Hydrochloric Acid
Heptafluorobutyric Acid Anhydride

A.3 Analytical Standards

The following standards are used to analyze for triflumizole and its degradates. Standards should be stored frozen. Standards can be obtained from Uniroyal Chemical Company. Structures for these standards are shown in Figure 1 (see Appendix A for certificates of analysis).

Analytical Standard	Lot Number	Purity
Triflumizole (1-[N-(4-chloro-2-(trifluoromethyl)phenyl)-2-propoxyacetimidoyl]imidazole) (CAS No. 68694-11-1)	AC-1261-146	98.5%
FA-1-1 (2-amino-5-chlorobenzotrifluoride)	61318-C	97.8%
FD-1-1 (4-chloro-2-trifluoromethyl-propoxyacetanilide)	82-01-1	99.0%
FM-6-1 (N-[4-chloro-2-(trifluoromethyl)phenyl]imino-2-propoxyethylamine)	576-SY	99.6%
FA-1-1 HFBA derivative	979P30	100.0%

B. SAFETY AND HEALTH

This method should be performed by trained chemical personnel. Hazards associated with chemicals used in this analytical method are shown in the MSDS sheets in Appendix K.

C. ANALYTICAL METHOD

C.1 Principle of the Method

The analytical method for triflumizole, and three potential soil degradates, involves chemical hydrolysis to a common aniline (2-amino-5-chlorobenzotrifluride; FA-1-1), followed by derivatization with heptafluorobutyric acid anhydride (HFAA) to form the corresponding hepafluorobutyrylanilide (HFBA), to enhance the GC/ECD sensitivity.

The scheme outlining conversion of triflumizole and metabolites to FA-1-1 and conversion of FA-1-1 to the HFBA derivative is shown in Figure 2.

C.2 Types of Soil

This method is predicted to be applicable to most soil types. In Uniroyal Chemical study No. RP-96030, soils from a California USA location were used and the composition was a sandy loam from 0 to 30 inch depth and a loam at 30 to 36 inch depth. In Uniroyal Chemical study No. RP-97023, soils from a North Carolina USA location were used and the composition was a sandy loam at 0 to 6 inch depth and a sandy clay loam at 6 to 36 inch depth.

C.3 Sample Processing

All soil samples were stored frozen before processing. Processing consisted of allowing soil in bags to thaw slightly. Bags were pounded with a rubber mallet and contents mixed in a bag. Fifty-gram aliquots were removed for soil analysis. Ten-gram aliquots were removed for dry/wet weight ratio determination: samples were dried in an oven at 105°C overnight and allowed to cool in a desiccator prior to weighing. Samples were further oven dried for approximately 2 hours and cooled prior to reweighing in order to ensure constant weight. The balance of the soil samples was stored frozen.

C.4 Soil Extraction Method (see Figure 3 for Flow Chart)

1. Weigh 50 g of soil into a 500 ml-flat-bottomed flask. Fortify with the appropriate level of triflumizole or metabolite, if required. Add 200 ml 1 N NaOH prepared in HPLC grade water and add 1 drop of antifoam (continue immediately to next step).

2. Add 10 ml Optima-grade hexane to the reservoir of a Nielsen-Kryger steam distillation apparatus (Ace Glass 6555-40, see Figure 4). Attach the sample flask after applying a small amount of silicone vacuum grease to the ground glass fitting. Place a glass wool plug laced with approximately 1 ml hexane in the top of the steam distillation apparatus. Circulate cold water through the condenser. Heat the sample to boiling on a hot plate.

3. Allow the distillation of FA-1-1 to proceed for approximately 1 hour, until the level of aqueous distillate approximately matches the level of hexane. Turn off the heat and allow the system to cool slightly so that hexane is no longer refluxing. Collect the aqueous and hexane phases into a separatory funnel. Use a few ml of hexane to wash the glass plug; the "wash" collects in the reservoir of the condenser. Draw-off the hexane wash and combine with the previously collected hexane/water phase in the separatory funnel.

4. Add an additional 5 ml hexane to the collection reservoir and continue the distillation until approximately 5 ml water collects. Turn off the heat and allow to cool slightly. Draw off the hexane and water into the separatory funnel. Wash the interior of the condenser with a few ml of hexane and again combine in the separatory funnel.

5. Draw off the lower aqueous phase from the separatory funnel and discard. *Ensure that no water remains.* Transfer the hexane layer, plus washings to a 25 ml volumetric flask. Bring the sample volume to the mark with hexane, stopper and invert to mix thoroughly.

6. Transfer 5 ml of solution from the volumetric flask to a clean, dry glass tube and add 50 µl HFAA (heptafluorobutyric acid anhydride, Pierce). Cap, mix well and allow to stand 10 minutes.

7. Decompose excess HFAA by washing the hexane solution with 5 ml of saturated sodium bicarbonate solution. Allow to stand at least 5 minutes. Transfer the hexane phase to a new tube and repeat the sodium bicarbonate wash step. Transfer the hexane phase to another new tube and wash with approximately 5 ml HPLC grade water and allow to stand for at least 5 minutes. Repeat the water rinse step, if necessary. At this point the hexane phase should not smell of heptafluorobutyric acid.

8. Transfer a 1-ml aliquot of the washed hexane phase to a GC vial for analysis. In the case of the 1.0 ppm or 0.5 ppm fortified samples, transfer 1 ml to a 10 ml volunetric flask and dilute to 10 ml with hexane. Mix well. Transfer 1 ml of the dilution to a GC vial for analysis. For GC/ECD analysis, the concentration of samples and standards (FA-1-1 HFBA derivative) should not exceed 5 ng/µl.

9. Dispense approximately 1-ml aliquots to GC vials and cap for GC/ECD using a DB-1701 or DB-225 (J & W) capillary column. Also dispense approximately 1 ml aliquots of reference standard (FA-1-1 HFBA derivative) dilutions: 0.005 ppm, 0.010 ppm, 0.025 ppm, 0.050 ppm, 0.1 ppm, 0.25 ppm (include all appropriate concentrations).

   Mass Conversion Factors:
   Triflumizole to FA-1-1 HFBA = 1.13
   FA-1-1 to FA-1-1 HFBA = 2.0
   FM-6-1 to FA-1-1 HFBA = 1.33
   FD-1-1 to FA-1-1 HFBA = 1.32

   The analyte was identified by the coincidence of its retention time with the reference standard, and quantified by integration of the peak area or measurement of peak height using calibration curve slope and

intercept parameters, (see Methods of Calculation section). Gas chromatography Method 1 was used in the first replicate of FA-1-1 method validation. Gas chromatography Method 2 was developed to avoid matrix interference and was used in the analysis of triflumizole, FM-6-1 and FD-1-1 method validation samples, and the second FA-1-1 method validation.

Transport Spike Soil Fortification Procedure

Solutions of triflumizole, FA-1-1, FD-1-1 and FM-6-1 were prepared at the analytical laboratory to contain 25 µg/ml and 50 µg/ml. The latter solutions were shipped on dry ice, along with samples of acetone and hexane, syringes and spiking instructions, to the field lab. The solutions were used to fortify control soil at 0.0 ppm, 0.05 ppm and 0.1 ppm. Fortified soil samples were shipped frozen to the analytical laboratory where they were stored frozen until analysis.

C.5 Preparation of Standards

Stock solutions of triflumizole, FA-1-1, FM-6-1 and FD-1-1 were prepared using the formula described in the "Methods of Calculations" section. Stock solutions of triflumizole, FD-1-1 and FM-6-1 were each prepared in acetone at a concentration of 1000 µg/ml. For 100 µg/ml solutions, the 1000 µg/ml stock solutions were diluted 10-fold with acetone (i.e., 10 ml of the 1000 µg/ml diluted with 90 ml of acetone). Next, dilution of 5 ml of each of the 100 µg/ml solutions was made with acetone to produce 100 ml of corresponding 5 µg/ml solutions. Dilution of 20 ml of each of the 5 µg/ml solutions with acetone produced 100 ml of 1 µg/ml solutions. A 1000 µg/ml stock solution of FA-1-1 was prepared in hexane. Dilution of 5 ml of the latter 1000 µg/ml solution was made with hexane to produce 50 ml of 100 µg/ml solution. Next, dilution of 5 ml of the 100 µg/ml solution was made with hexane to produce 50 ml of a 10 µg/ml solution. Dilution of 12.5ml of the 10 µg/ml solution was made with hexane to produce 25 ml of a 5 µg/ml solution. Lastly, 5 ml of the 5 µg/ml solution was diluted with hexane to produce 25 ml of a 1 µg/ml solution. Microliter syringes, volumetric pipettes and volumetric flasks were used throughout.

C.6 Preparation of FA-1-1 HFBA Derivative and Linearity Standards (Calibrants)

FA-1-1 HFBA derivative was prepared previously as described by Baker et al. (Ref. 1): A solution of FA-1-1 (62 mg/ml hexane) was transferred to a separatory funnel containing 10 ml of hexane. Heptafluuorobutyric acid anhydride (1 ml) was mixed with the FA-1-1 solution and the stoppered funnel was allowed to stand at room temperature in a fume hood for 30 minutes. The hexane solution was washed several times with water, once with saturated sodium carbonate solution, then water, 0.1N HCl and finally twice more with water. The hexane phase was filtered through anhydrous sodium sulfate into a tared flask. A small aliquot was removed for TLC on

a Silica GF microplate and developed with hexane:methylene chloride (1:1). A single UV absorping spot was observed (R_f 0.55). The rest of the solution was reduced to dryness; weight of product was 97 mg (78% yield).

A 1 mg/ml stock solution of FA-1-1 HFBA derivative (calibrant) was prepared by dissolving 21.68 mg of the above sample in 21.68 ml of hexane. Serial dilutions of the latter were prepared in hexane. Using the 2.5 µg/ml HFBA-derivatized FA-1-1 stock standard, the following linearity calibrants were prepared in volumetric flasks:

FA-1-1 HFBA Standard Dilution (in hexane)

0.25 µg/ml = 5 ml of 2.5 µg/ml FA-1-1 HFBA diluted to 50 ml
0.10 µg/ml = 20 ml of 0.25 µg/ml FA-1-1 HFBA diluted to 50 ml
0.05 µg/ml = 25 ml of 0.10 µg/ml FA-1-1 HFBA diluted to 50 ml
0.025 µg/ml = 25 ml of 0.05 µg/ml FA-1-1 HFBA diluted to 50 ml
0.01 µg/ml = 20 ml of 0.025 µg/ml FA-1-1 HFBA diluted to 50 ml
0.005 µg/ml = 25 ml of 0.01 µg/ml FA-1-1 HFBA diluted to 50 ml

A calibration curve was generated with each sample set to determine linearity and to quantitate triflumizole, FA-1-1 and FD-1-1 residues as FA-1-1 HFBAA equivalents. The latter were expressed as triflumizole equivalents in analytical samples.

C.7 Soil Method Validation Fortification Procedure

Fortification of untreated soil samples with triflumizole, FA-1-1, FM-6-1 and FD-1-1 was performed to validate the analytical method. A portion (50 g) of untreated soil was fortified to 0.01 ppm triflumizole (0.5 ml of 1.0 µg/ml triflumizole). Similarly, the 0.05 and1.0 ppm triflumizole fortification levels were achieved by addition of 0.5 ml of 5.0 µg/ml triflumizole stock and 0.5 ml of 100 µg/ml triflumizole stock, respectively. Fortification of untreated soil samples with FA-1-1 was conducted in the same manner with the 1.0 µg/ml FA-1-1, 5.0 µg/ml FA-1-1 and 100 µg/ml FA-1-1 stock solutions. Method validations for triflumizole and FA-1-1 were conducted twice. Fortification of untreated soil samples with FM-6-1 at 0.01 ppm and 1.0 ppm were achieved by addition of 0.5 ml of the 1.0 µg/ml FM-6-1 and 100 µg/ml FM-6-1 stock solutions , respectively. Finally, untreated soil samples were fortified with FD-1-1 at 0.01 ppm and 1.0 ppm by addition of 0.5 ml of the 1.0 µg/ml FD-1-1 and 100 µg/ml FD-1-1 stock solutions to 50 g soil samples. FM-6-1 and FD-1-1 validations were conducted once.

C.8 Soil Analytical Sample Fortification Procedure

Fortification of untreated soil with triflumizole was performed to monitor method recoveries for each treated sample set during soil dissipation studies conducted at two

locations in the USA. The level of fortification was varied during the studies according to the anticipated level of residue in soil samples (e.g. according to soil depth, time after application etc.) Typically two 50-g portions of untreated soil were fortified to 0.1 ppm, 0.5 ppm or 1.0 ppm. In addition, several sample sets included FA-1-1, FD-1-1 or FM-6-1 fortifications.

C.9 Gas Chromatography Method and Instrumentation

Gas Chromatography Analysis of FA-1-1 HFBA

Model No. 5890 Series IIA Hewlett Packard Gas Chromatograph (GC) equipped with Electron Capture Detector (ECD)

Method 1:

Column:	J & W Scientific DB 1701 Capillary Column 30m X 0.32mm i.d. X 0.25 µm film thickness
Flow Rate:	Carrier Gas = 3 ml/minute helium Make-up Gas = 30 ml/minute 5% Methane/95% Argon(P5)
Injector Temperature:	250°C
Detector Temperature:	325°C
Oven Temperature:	Initial Temperature: 125°C, 5 min. Ramp: 125°C to 140°C at 2°C/minute 140°C to 240°C at 30°C/minute 240°C for 2 minutes
Injection Volume:	1 µl; by Hewlett Packard 7673A Autosampler
Retention Time:	7.8 to 7.9 minutes

Method 2:

Column:	J & W Scientific DB 225 Capillary Column 15 m X 0.53mm i.d. X 1.0 µm film thickness
Flow Rate:	Carrier Gas = 3 ml/minute helium Make-up Gas = 30 ml/minute:5% Methane/95% Argon(P5)
Injector Temperature:	225°C
Detector Temperature:	300°C

Oven Temperature: Initial Temperature: 80°C, 2 minute
Ramp: 80°C to 125°C at 2°C/minute
125°C for 1 minute
125°C to 200°C at 30°C/minute
200°C for 2 min.

Injection Volume: 2 µl; by Hewlett Packard 7673A Autosampler

Retention Time: 19.9 to 20.0 minutes

Method 3:

Column: J & W Scientific DB 225 Capillary Column
30m X 0.53mm i.d. X 1.0 µm film thickness

Flow Rate: Carrier Gas = 2 ml/minute helium
Make-up Gas = 50 ml/minute 5% Methane/95% Argon(P5)

Injector Temperature: 225°C

Detector Temperature: 300°C

Oven Temperature: Initial Temperature: 105°C, 2 min.
Ramp: 105°C to 125°C at 1°C/minute
125°C for 1 minute
125°C to 200°C at 30°C/minute
200°C for 12 min.

Injection Volume: 2 µl; by Hewlett Packard 7673A Autosampler

Retention Time: 18.1 to 22.8 minutes

Method 4:

Column: J & W Scientific DB 1701 Capillary Column
30m X 0.32mm i.d. X 0.25 µm film thickness

Flow Rate: Carrier Gas = 1 ml/minute helium
Make-up Gas = 50 ml/minute nitrogen

Injector Temperature: 250°C

Detector Temperature: 325°C

Injection Volume: 1 µL

Oven Temperature: Initial Temperature: 115°C, 5 min.
Ramp: 115°C to 130°C at 2°C/minute
130°C for 1 minute
130°C to 280°C at 30°C/minute
280°C for 7 min.

Retention Time: approx. 7 minutes

GC/MS Analysis of FA-1-1 HFBA

GC/MS analysis of FA-1-1 HFBA calibrant and extracts containing FA-1-1 converted to the HFBA derivative was conducted on a HP 5971A MSD instrument using modified GC method 1 (see below) to give a retention time for the analyte of approximately 7-8 minutes. Total ion chromatograms and full mass spectra were generated.

Oven Temperature: Initial Temperature: 80°C, 1 min.
Ramp: 80°C to 125°C at 5°C/minute
125°C for 1 minute
125°C to 140°C at 2°C/minute
140°C to 240°C at 30°C/minute
240°C for 2 min.

GC/MS characterization of the FA-1-1 HFBA standard was also conducted on an HP 5971A MSD instrument using a simplified method 1: Injector temperature was 250°C, and detector temperature was 270°C. Initial oven temperature was 80°C for 1 minute followed by a temperature program at 10°C/minute to 260°C; the temperature was held at 260°C for 5 minutes. Retention time of FA-1-1 HFBA was approximately 5.7 minutes. The GC/MS Analysis of FA-1-1 HFBA is presented in Appendix B.

D. SAMPLE BRACKETING

A typical injection sequence for validation samples was: hexane solvent blank, 0.005 µg/ml FA-1-1 HFBA standard, reagent blank sample, untreated (control) sample, 0.01 µg/ml standard, 0.01 µg/g fortified soil, 0.01 µg/g fortified soil, 0.025 µg/ml standard, 0.05 µg/g fortified soil, 0.05 µg/g fortified soil, 0.1 µg/ml standard, etc.

E. POTENTIAL INTERFERENCES

This method could have interferences from other pesticides that might elute with similar retention times. The soil history should be considered in this respect and a confirmatory technique should be used if a problem is suspected.

F. CONFIRMATORY TECHNIQUES

Extracts from soils were used for GC/MS analysis to confirm the presence of FA-1-1 (as HFBA). Portions of the distilled FA-1-1 in hexane (as HFBA derivative, generated as described in the soil analytical method) were gently evaporated under a N_2 stream to approximately one tenth of original volumes. Concentrated samples, along with the FA-1-1 HFBA standard (0.01 mg/ml hexane) were subjected to GC/MS analysis using an HP 5971 mass selective detector.

G. TIME REQUIRED FOR ANALYSIS

Time required for a sample set, where a sample set consists of eight (8) to ten (10) matrix samples:

Extraction by steam distillation, partition, derivatization, clean-up etc. takes approximately 8 hours.

GC analysis takes approximately 10-11 hours.

Data entry, spreadsheet generation and evaluation takes 2 hours.

TOTAL = approximately 20-21 hours.

H. MODIFICATION OR POTENTIAL PROBLEMS

None

I. CALCULATIONS

Preparation of Stock Standards

$$\text{Volume of Solvent (ml)} = \frac{(W)}{(FC)} \times \frac{P^*}{100}$$

where W = micrograms of neat standard
P^* = chemical purity of neat standard (correction made in tables, not during preparation of stock standard)
FC = final concentration (µg/ml)

Recoveries

The recoveries of triflumizole , FA-1-1, and FD-1-1 from fortified soil samples were calculated as follows, where the appropriate analyte would be substituted for "triflumizole" in the formula:

Linear regression formula from calibration curve y = mx + b (generated with Excel® program)

$$x = \text{nanogram FA-1-1 HFBA} = \frac{y-b}{m}$$

where y = sample peak area/height
b = calibration intercept
m = slope

$$\text{ppm triflumizole} = \frac{\text{ng triflumizole}}{2\ \mu\text{l inj.}} \times \frac{25\ \text{ml} \times \text{D.F}}{\text{Sample Wt (g)}} \times \frac{1\ \mu\text{g}}{1000\ \text{ng}} \times \frac{1000\ \mu\text{l}}{1\ \text{ml}}$$

where ng triflumizole = ng FA-1-1 HFBA ÷ M.W. conversion factor
M.W. conversion factor = M.W. FA-1-1 HFBA ÷ M.W. triflumizole
(see p. 13 for conversion factors)

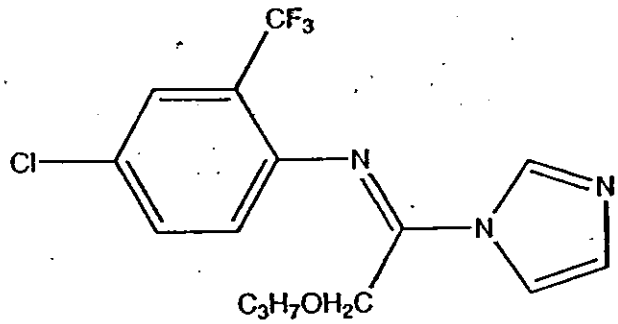
D.F. = dilution factor

Percent Recovery =

$$\frac{\text{Conc of Triflumizole Fortified Sample (µg/g) - Conc. of Triflumizole Control Sample (µg/g)}}{\text{Triflumizole Fortification Level (µg/g)}} \times 100$$

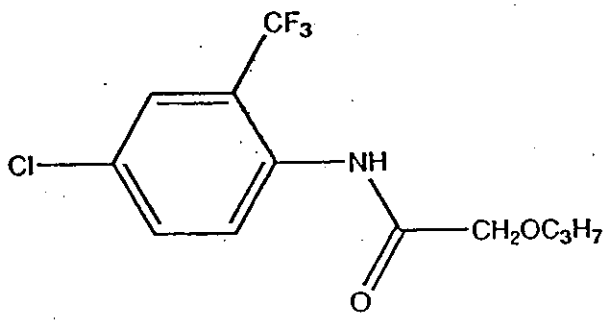
An acceptable percent recovery (70 –120%) of the triflumizole from soil demonstrated validity of the analytical method and determined the limit of quantitation. The actual ppm triflumizole in soil was corrected for soil moisture content as follows:

$$\text{ppm triflumizole in dry soil} = \text{ppm triflumizole in wet soil} \times \frac{\text{wet soil weight (g)}}{\text{dry soil weight (g)}}$$



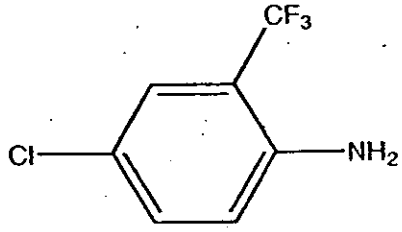
Triflumizole

(*E*)-[1-[1[[4 chloro-2-(trifluoromethyl)phenyl]imino]-2-propoxyethyl]-1*H* imidazole]



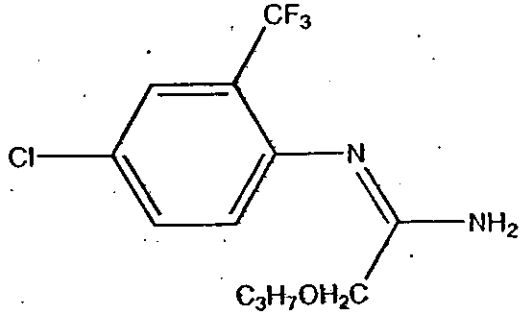
FD-1-1

4-chloro-2-trifluoromethyl-propoxyacetanilide



FA-1-1

2-amino-5-chlorobenzotrifluoride



FM-6-1

N-[4-chloro-2-(trifluoromethyl)phenyl]imino-2-propoxyethylamine

Figure 1. Chemical Structures for Triflumizole and its Degradates in Soil.

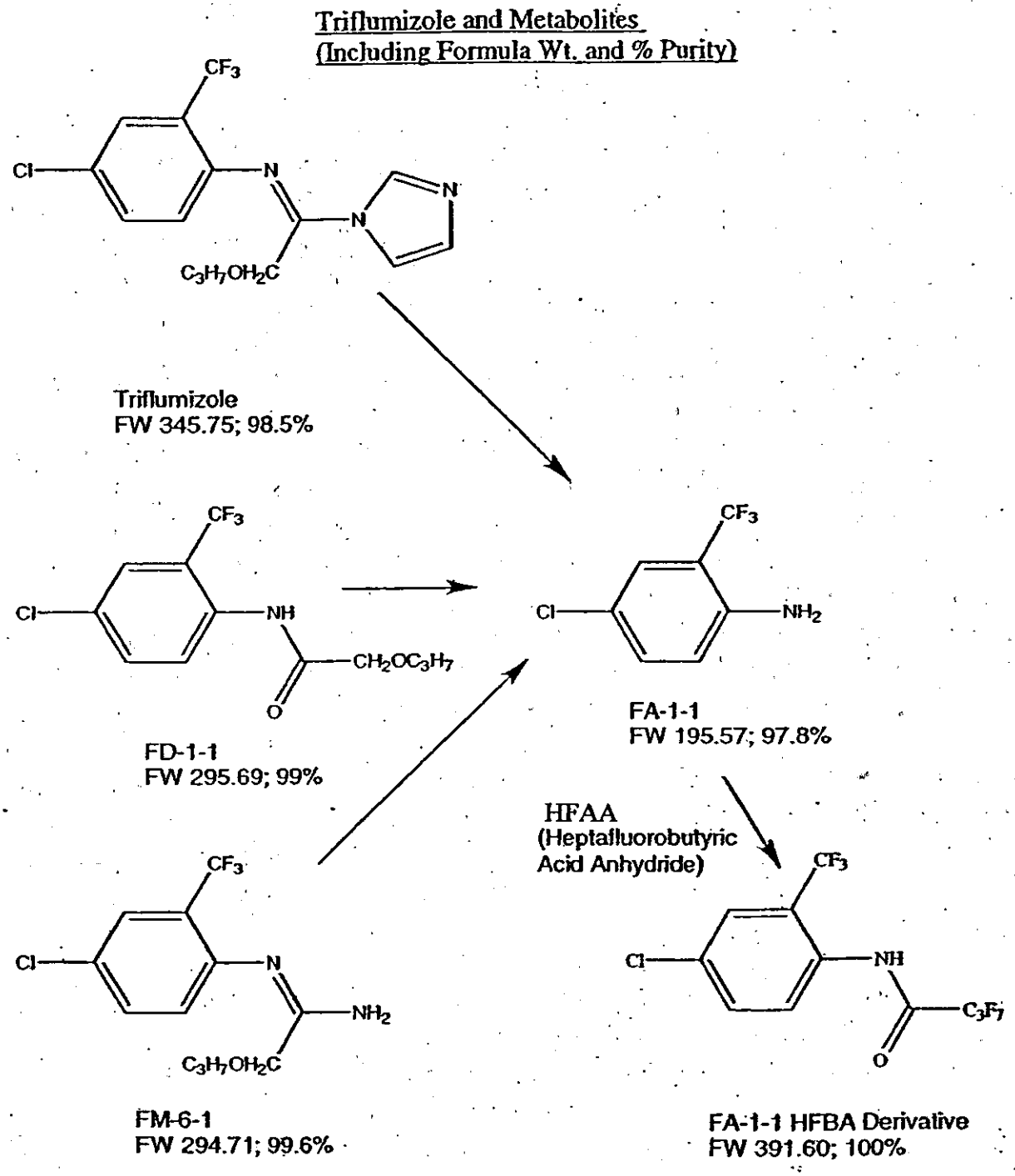


Figure 2. Scheme Showing Conversion of Triflumizole and Degradates to FA-1-1 and Conversion of FA-1-1 to the HFBA Derivative.

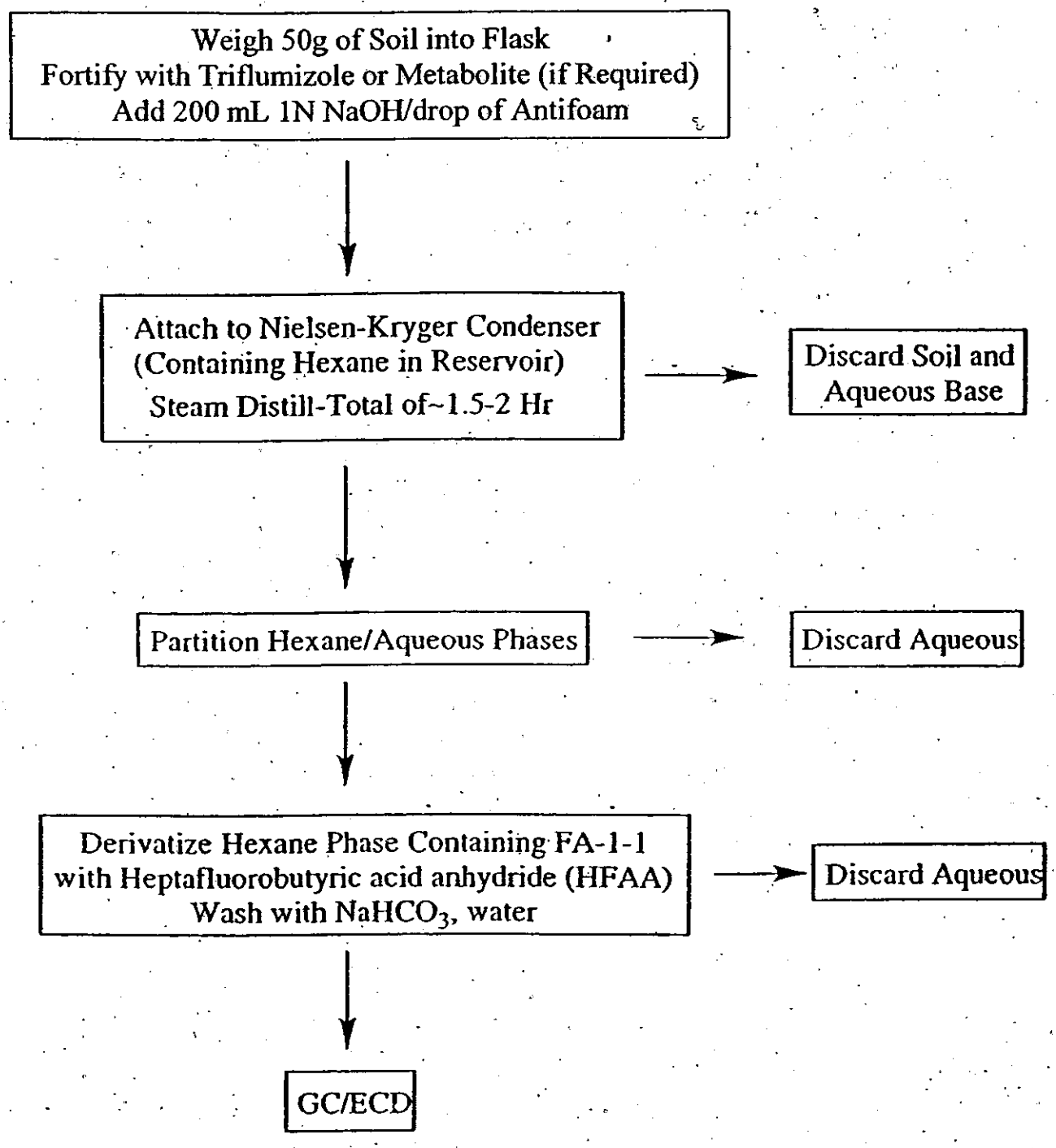
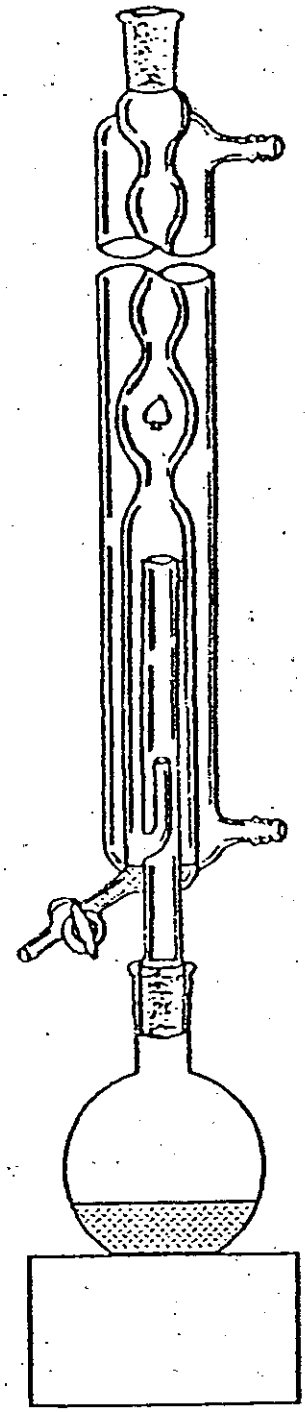


Figure 3. Flow Chart Summarizing Soil Analytical Method.



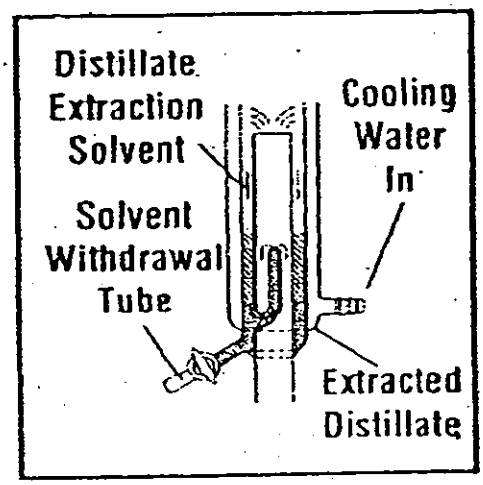


Figure 4. Soil Extraction Procedure Using a Nielsen-Kryger Distillation Apparatus.