Insecticides, Fipronil: Method of Analysis for the Determination of Fipronil and its Metabolites in Water

I. Introduction

A. Scope

An analytical method is described for the analysis of fipronil and its metabolites in water as defined in the Pesticide Assessment Guidelines, Subdivision N and 40 CFR 158.290.

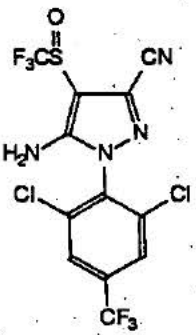
B. Principle

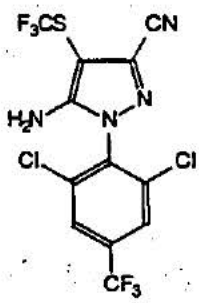
Residues of fipronil and its non-polar metabolites MB 45950, MB 46136, MB 46513 and RPA 200766 are extracted from well water, surface water, and rice paddy water with toluene. The toluene phase is brought up to the appropriate volume and prepared for analysis. A trace enrichment of the polar metabolite RPA 104615 is accomplished by passing the aqueous phase through a C18-ENV Sep-Pak Plus cartridge. The methanol eluate is evaporated to dryness and then hydrolyzed at ~70°C to MB 45897 with sulfuric acid in acetonitrile/methanol. After neutralization, evaporation of the acetonitrile and addition of water, the residues are partitioned into toluene. The toluene phase is then brought up to the appropriate volume and prepared for analysis. Quantification of fipronil and its metabolites is accomplished by gas chromatography using a Ni^{63} electron capture detector.

C. Method Limits

The minimum method detection limits (MDL) and limits of quantification (LOQ) for fipronil, MB 45950, MB 46136, MB 46513, RPA 104615 and RPA 200766 (as MB 45897) in each substrate have not been determined. This information will be obtained from the subsequent validation study. The target level for LOQ is 1 ppb for fipronil and its metabolites in water.

D. Chemical Structures


MB 46030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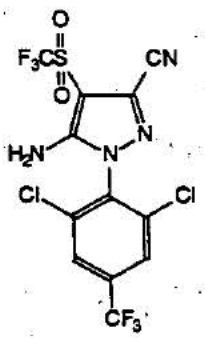
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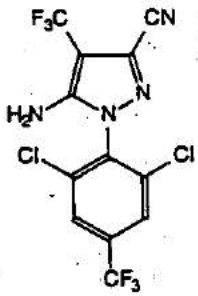
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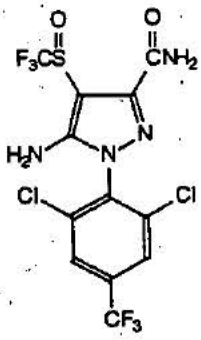
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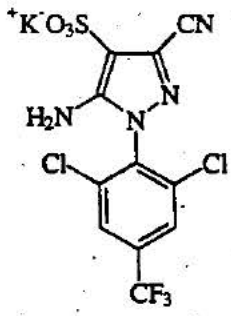
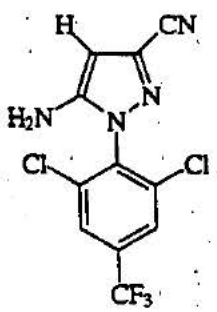

RPA 104615


MB 45897

II. Materials

Reagents and Solvents were used as received from supplier, unless otherwise noted.

Equivalent reagents and equipment may be substituted where appropriate.

A. Reagents

1. Sodium Chloride GR, crystals, EM Science, Cat. No. SX0420-1, or equivalent
2. Sulfuric acid, Solution, 10% (v/v), VWR, Cat. No. VW3258-1, or equivalent
3. Potassium Carbonate, crystals, EM Science, Cat. No. SX0420-1, or equivalent

B. Solvents

1. Acetonitrile UV, B & J High Purity Solvents, Burdick & Jackson, Cat. No. 015-4, or equivalent
2. Acetone, B & J Chrompure HPLC Solvent, Burdick & Jackson, Cat. No. CP80100-4, or equivalent
3. Methanol, B & J Chrompure HPLC Solvent, Burdick & Jackson, Cat. No. CP80150-4, or equivalent
4. Toluene, B & J Brand, Burdick & Jackson, VWR Cat. No. BJ347-4, or equivalent
5. Water, Milli-Q, or equivalent

C. Solutions

1. 5% Potassium Carbonate Solution

   Add ~5 g of potassium carbonate to a 250mL Erlenmeyer flask. Add ~95mL of Milli-Q water and mix thoroughly.

D. Equipment

1. Aluminum Crimp-Top Seal, 11 mm TFE/RUB Septum, Sun Brokers, Inc., Cat. No. 200 100, or equivalent
2. Amber bottles, 2 oz. and 4 oz., screw-capped, VWR, Cat. No. 16153-102, Cat. No. 16153-135, or equivalent

3. Analytical Balance, Mettler PM2000, or equivalent top-loading balance

4. Autosampler Vials, 1 mL, clear, Wheaton, Cat. No. 223682, or equivalent

5. Beakers, 250mL, VWR, Cat. No. 13910-201, or equivalent

6. Boiling Flask, Flat-bottom, 125-mL, VWR, Cat. No. 29114-023, or equivalent

7. Bransonic Ultrasonic Cleaner, Branson 5210, VWR Cat. No. 21810-212, or equivalent

8. C18-Environmental, Sep-Pak Plus cartridges, Waters, Part No. WAT023635, *no substitute*

9. Capillary Column, DB1701, 15 m X 0.32 mm i.d., 0.25 µm film thickness, J & W Scientific, Cat. No. 123-0712, or equivalent

10. Centrifuge, Marathon 10K, Fisher Scientific, Type 2510, or equivalent

11. Centrifuge Tubes, Blumax, VWR Cat. No. 21008-951, or equivalent

12. Disposable Pasteur Pipets, VWR, Cat. No. 14673-043, or equivalent

13. Erlenmeyer flasks, appropriate sizes, VWR, eg. Cat. No. 29137-426, or equivalent

14. Glasswool, VWR, Cat. No. 32848-003, or equivalent

15. Graduated Cylinders, calibrated to deliver, appropriate sizes, VWR, eg. Cat. No. 34795-056, or equivalent

16. Graduated Mixing Cylinders, calibrated to contain, 100 mL, VWR, Cat. No. 24762-332, or equivalent

17. Hewlett Packard 5890 Series II GC equipped with ^{63}Ni detector (refer to Section V of this document for details)

18. Hewlett-Packard 7673A Autosampler Syringes, Hamilton, 5µL, VWR Cat. No. 60373-405, or equivalent

19. Pear-Shaped Flasks, 25mL, VWR, Cat. No. KT294250-0025

20. Reservoir, 75-mL, Varian, Cat. No. 1213-1012 or equivalent

21. Rotary Evaporator, Buchi, R-124, or equivalent

22. Separatory Funnels, 250 mL, VWR, Cat. No. 30356-664, or equivalent

23. Standard Taper Clamps, Wheaton, VWR Cat. No. 21734807, or equivalent

24. Volumetric Flasks, appropriate sizes, class A, VWR, eg. Cat. No. 29619-610, or equivalent

25. Volumetric Pipettes, appropriate sizes, class A, VWR, eg. 53046-269

26. Optional: Culture Tubes, screw-cap (16mm x 125mm), VWR, Cat. No. 60827-533, or equivalent

27. Optional: (a) TurboVap® LV Evaporator, Zymark, or equivalent or b) Meyer N-EVAP Analytical Evaporator, Model No. 111, or equivalent

28. Optional: Gilson pipettors, appropriate sizes, Markson

E. Analytical Standards

Analytical Standards available from Rhône-Poulenc Ag Company

1.	Fipronil (MB 46030):	5-amino-1-(2,6-dichloro-4-trifluoromethylphenyl)-3-cyano-4-trifluoromethanesulphinylpyrazole
2.	MB 45897:	5-amino-1-(2,6-dichloro-4-trifluoromethylphenyl)-3-cyanopyrazole
3.	MB 45950:	5-amino-1-(2,6-dichloro-4-trifluoromethylphenyl)-3-cyano-4-trifluoromethylthiopyrazole
4.	MB 46136:	5-amino-1-(2,6-dichloro-4-trifluoromethylphenyl)-3-cyano-4-trifluoromethylsulphonylpyrazole

5. MB 46513: 5-amino-1-(2,6-dichloro-4-trifluoromethylphenyl)-3-cyano-4-trifluoromethylpyrazole

6. RPA 200766: 5-amino-3-carbamoyl-1-(2,6-dichloro-4-trifluoromethyl-phenyl)-4-trifluoromethylsulphinylpyrazole

7. RPA 104615: 5-amino-3-cyano-1-(2,6-dichloro-4-trifluoromethyl-phenyl)pyrazole-4-sulfonic acid, potassium salt

III. Fortification and Calibration Standard Solutions

A. Preparation

The stated concentrations of standard solutions should be adjusted to account for the purity of the neat solid standards. After preparation, standards should be transferred from the volumetric flasks into screw-capped amber bottles to prevent possible photodegradation. Store standard solutions in the refrigerator at less than 10 °C when not in use.

The following is provided as an example of how standard solutions may be prepared. Other concentrations may be used as appropriate.

1. For the fortification standards:

   1.1 Weigh 0.1000 g (±0.1 mg) of MB 45950, MB 46513, MB 46030, MB 46136, RPA 104615 and RPA 200766 individually into 100 mL volumetric flasks. Dissolve each in methanol and mix well. Dilute to final volume with methanol. The concentration of each standard is ~1000 µg/mL.

   1.2 Withdraw a 10.0 mL aliquot from each of the ~1000µg/mL individual standards and add to a 100 mL volumetric flask. Dilute to final volume with methanol. The concentration of this standard is ~100µg/mL for each analyte in the solution. (Note: The analytical standards may be diluted and used separately for fortification and individual determinations.)

1.3 Withdraw a 10.0 mL aliquot from the ~100µg/mL mixed standard and add to a 100 mL volumetric flask. Dilute to volume with methanol. The concentration of this standard is ~10µg/mL for each analyte in methanol.

1.4 By further dilution of the ~10µg/mL mixed standard with methanol, prepare a series of standards to serve as fortification standards.

2. For the calibration standards:

2.1 Weigh 0.1000 g (±0.1 mg) of MB 45897, MB 45950, MB 46513, MB 46030, MB 46136 and RPA 200766 individually into 100 mL volumetric flasks. Dissolve each in acetonitrile and mix well. Dilute to final volume with acetonitrile. The concentration of each standard is ~1000µg/mL.

2.2 Withdraw a 10.0 mL aliquot from each of the ~1000µg/mL individual standards and add to a 100 mL volumetric flask. Dilute to final volume with toluene. The concentration of this standard is ~100µg/mL for each analyte in the solution.

2.3 Withdraw a 10.0 mL aliquot from the ~100µg/mL mixed standard and add to a 100 mL volumetric flask. Dilute to volume with toluene. The concentration of this standard is ~10µg/mL for each analyte in toluene.

2.4 By further dilution of the ~10µg/mL mixed standard with toluene, prepare a series of standards to serve as calibration standards.

B. Stability

1. Calibration standard and fortification solutions of fipronil and its metabolites (MB 45940, MB 46136, MB 46513 and RPA 200766) in acetonitrile have been shown to be stable for periods of approximately three months when stored within a temperature range of 0°C to +4°C ("Fipronil: Magnitude of Residues in/on Cottonseed and Gin Trash Under a Modified Application Scenario", Study No. US95V10R, Robert S. Plaisance, Dec. 10, 1996; "Fipronil: Validation of Method of Analysis for Fipronil and its Metabolites in Field Corn", Study No. EC-93-236, Suvit Upalawanna, Aug. 27, 1993 (MRID #43323401). Standard solution stability was demonstrated by preparing a new aliquot of the fifty part per billion

fortification solution from the one-hundred microgram per milliter intermediate stock solution and comparing it to the previous fifty part per billion fortification solution. The results were compared by area count and showed variations of less than ±10% which is the limit normally accepted for gas chromatography. The exception is RPA 200766, which indicated an approximate fifteen percent difference in response (Study No. US95V10R).

IV. Methods of Analysis

The diamond symbol (♦) indicates a stopping point in the method.
Overnight storage at room temperature (~15°C - ~30°C) *is suitable, however, a freezer is recommended for longer time periods. Samples should be allowed to warm / thaw prior to use.*

A. Well Water, Surface Water and Rice Paddy Water

1. Weigh ~100.00 g of water into a 250-mL beaker (or any other appropriate vessel) and transfer it to a 250-mL separatory funnel. Mix sample prior to weighing.

2. Perform sample fortification at this point if appropriate.

3. Optionally, prepare a"Reference Spike" by pipetting the same volume and the same standard solution used for the sample fortification into an appropriately sized volumetric flask. Dilute to volume with toluene. Store reference spikes in the refrigerator. *The reference spikes can be analyzed along with those samples available from step 22.*

4. Add ~3g sodium chloride to the separatory funnel and shake to dissolve.

5. Add ~10mL toluene and shake gently for ~1 minute.

6. Allow ~5-10 minutes for the emulsion to clear substantially. After separation, drain the aqueous layer into an appropriately sized Erlenmeyer flask. Drain the upper toluene layer into a 50mL calibrated centrifuge tube.

7. Pour the aqueous phase back into the above separatory funnel. Rinse the flask with ~10mL toluene and add to the separatory funnel. Shake gently for ~1 minute.

♦8. Allow ~5-10 minutes for the emulsion to clear substantially. After separation, drain the aqueous layer into the same Erlenmeyer flask used in step 6.

Save the aqueous layer for the trace enrichment procedure, step 11. Drain the upper toluene layer into the same 50mL calibrated centrifuge tube used in step 6.

♦9. Centrifuge the tube for ~10 minutes at ~2000rpm or at a moderate setting (50-60). Remove any water that has settled with a pipette and discard.

Bring to the appropriate volume (eg. for rice paddy water test samples, bring to 20mL) with toluene and mix thoroughly. Transfer the toluene to a vial prior to gas chromatography and analyze for fipronil and its non-polar metabolites MB 45950, MB 46513, MB 46136 and RPA 200766. *The sample can be analyzed along with those available from step 22.*

10. Add a 75mL reservoir to a Waters C18-ENV Sep-Pak Plus Cartridge. Add a small pad of glass wool to the bottom of the reservoir. Condition with ~10mL methanol and then equilibrate with ~10mL water at a rate of ~2 drops/sec (~2mL/min). *Do not let the cartridge dry.*

11. Load the aqueous sample from step 8 onto the cartridge and elute at a rate of ~2 drops/sec (~2mL/min). *The flow rate is critical. Do not let the cartridge dry.* Discard the effluent.

12. Rinse the sample flask with ~10mL water and add to the cartridge. Elute the cartridge to dryness (in order to minimize the amount of water in the subsequent effluent) at a rate of ~2 drops/sec (~2mL/min). *The flow rate is critical.* Discard the effluent.

♦13. Place an Erlenmeyer flask under the cartridge. Elute off RPA 104615 with ~5mL methanol. Elute the cartridge to dryness at a rate of ~2 drops/sec (~2mL/min). *The flow rate is critical. The samples may be stored at room temperature* (~15°C - ~30°C) *overnight.*

14. Transfer contents to a 25-mL pear-shaped evaporation flask. Rinse the Erlenmeyer flask with acetonitrile. Evaporate to just dryness using a rotary evaporator with a ~40°C water bath. If necessary, add acetonitrile (~1-3mL) to help azeotrope off the residual water.

♦15. Dissolve the residue in ~200µL methanol. Sonicate for ~1 minute with swirling. Add ~5mL acetonitrile. Sonicate for ~1 minute with swirling, followed by ~5 minutes without swirling. *The samples may be stored at room temperature* (~15°C - ~30°C) *overnight.*

16. Add ~80µL of 10% sulfuric acid, stopper and whirlimix.

♦17. Stopper flask securely with a standard taper clamp and immerse in a water bath at ~70°C for ~ 1 hour. *The samples may be stored at room temperature* (~15°C - ~30°C) *overnight.*

♦18. Cool to ~ room temperature (~15°C - ~30°C) before adding ~1mL of 5% potassium carbonate solution. Shake vigorously. *The samples may be stored at room temperature* (~15°C - ~30°C) *overnight.*

19. Evaporate to ~0.5 - 1.0mL using a rotary evaporator with a ~40°C water bath. If necessary, add acetonitrile (~1-3mL) to help azeotrope off the residual water.

♦20. Add ~1mL water and then add an appropriate volume of toluene using a volumetric pipette. The volume of toluene can be adjusted depending upon the expected residue level. Shake vigorously and transfer the toluene to a vial prior to gas chromatography and analyze for RPA 104615 (as MB 45897).

B. General Notes

1. Alternatively, one can use a turbovap or an N-Evap to evaporate the samples from steps 14 and 19 after first transferring the samples to a screw cap culture tube (16mm x 125mm). However, this alternative procedure is much more time-consuming.

V. Gas Chromatography

A. Instrumentation

1. Gas Chromatograph:	Hewlett-Packard 5890 Series II GC, 7673 Autosampler, 18594B Sampler Controller, 3396A Integrator, Split/Splitless Injector, or an equivalent system
2. Detector:	Ni^{63} - Electron Capture Hewlett-Packard Model G1223A, G1224A, or equivalent
3. Data Acquisition:	Waters® 860 Data Capture System, or equivalent
4. Column:	J & W Scientific DB1701 15 m X 0.32 mm i.d., 0.25 µm film thickness

B. GC Conditions

1. Detector Make-Up Gas:	5% Methane in Argon, ~50-60 mL/min
2. Carrier Gas:	Helium, ~2-3 mL/min
3. Inlet Liner:	4-mm i.d. nominal volume 900 µl, borosilicate glass with silanized glass wool plug (HP part #5062-3587)
4. Injector Temperature:	280 °C
5. Detector Temperature:	300 °C
6. Oven Temperatures:	Initial: 50 °C, hold 1 minute Ramp 30 °C / min to 200 °C, hold 20 minutes Ramp 30 °C / min to 230 °C, hold 10 minutes Ramp 30 °C / min to 250 °C, hold 12 minutes
7. Injection Volume:	0.5 µL

8. Splitless injection with split vent off for 30 seconds.

C. General Notes

1. Gas flows set while oven at 50 °C.

2. Several standards should be injected prior to actual analysis using a new column or after the GC has set idle for any considerable length of time to condition and/or to remove any contaminants. Keep oven temperature at 100°C when not in use.

3. A gold plated seal is used at the interface of the glass liner and column. Hewlett-Packard Part No. 18740-20885.

4. Optionally, a gigabore liner is installed in detector make-up gas adapter. Hewlett-Packard Part No. 19233-20625.

5. The GC parameters are guidelines and can be optimized for the instrument and column actually used. Record the actual GC conditions used for data acquisition and include in report.

6. Due to matrix enhancement, peak responses of MB 46136 and RPA 200766 metabolites in the samples may be higher than in the standard solutions. Therefore, high recoveries of these metabolites may be observed. Replacement of the inlet liner, gold seal, and washer supporting the inlet sleeve beneath the injector (HP 5890 GC) as well as breaking off a bit of the column on the inlet side will help with this problem.

7. The GC sample vials must be rinsed with toluene or acetonitrile twice and air dried before use. Alternatively, one can rinse the GC vial twice with the solution to be analyzed, provided that there is enough sample. The GC vial caps must also be rinsed in a beaker with toluene and air dried.

8. The teflon stopcocks and stoppers on the separatory funnels must be removed from the separatory funnels and rinsed in a beaker with acetonitrile. They can then be washed with soap and water and rinsed with acetone or methanol as usual.

VI. Calculations

A. Calibration Curves

1. Linear regression should be used to generate calibration curves for all analytes. At least five different standard concentrations should be run with each set of samples. Standards should be interspersed with samples to compensate for any minor change in instrument response. Extracts should be diluted such that the peak areas obtained are within the area range between the lowest and highest standards injected.

2. Linear regression coefficients should be calculated from 'peak area' (or 'peak height') versus 'nanogram / mL injected'. Data from the analytical standards should be fit to the linear equation, $y = a + bx$.

   where: y = peak area or height
   a = calibration line intercept
   b = calibration line slope
   x = conc of analyte in inj soln

3. When the calculated values of the coefficient of determination (r^2) begin to decline, it is usually an indication that the GC inlet liner needs to be changed.

B. Quantification of Residues

1. Fipronil, MB 45897, MB 46513, MB 45950, MB 46136 and RPA 200766 can be quantified by comparision to the standard curves obtained from a linear regression analysis of the data.

2. The amount of RPA 104615 (MW 439.2) present in the extracts is calculated by quantifying the MB 45897 (MW 321.0) peak and then correcting for the molecular weight (conversion factor of 1.368).

•3. Equations

2.1 Concentration of analyte in sample in ppb (parts per billion).

$$z = (y - a) / b \times c / d$$

where: y = peak area (or height), response of analyte of interest

a = intercept of calibration line from linear regression (ng/mL)

b = slope of calibration curve from linear regression (response per ng/mL)

c = final volume of sample (mL)

d = sample weight (g)

z = conc of analyte in sample (ppb)

2.2 Percent recovery

$$\% \text{ recovery} = \frac{(\text{ppb found in fortified sample} - \text{ppb found in UTC})}{\text{actual fortification level in ppb}} \times 100\%$$