APPENDIX E

THE REVISED METHOD OF ANALYSIS

TITLE: Fipronil - Method of Analysis for Fipronil and its Metabolites in Field Corn Substrates and Processed Fractions

OBJECTIVE: To provide instruction for determination of fipronil and its metabolites in field corn substrates and Processed Fractions.
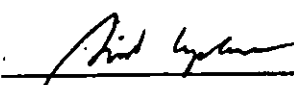
SCOPE: Fipronil residues consist of fipronil and its metabolites. The procedure describes the determination of these residues in field corn substrates and Processed Fractions.
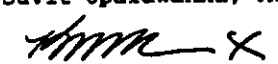
TEST SUBSTANCE Fipronil (MB 46030) and Its Metabolites, MB 45950, MB 46136, RPA 105048, and RPA 200766. All test and reference substances will be supplied by Rhône-Poulenc Ag Company.
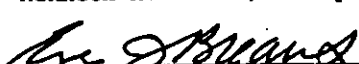
TEST SUBSTRATES: Field corn RAC substrates and processed fractions, as defined in Pesticide Assessment Guidelines, Subdivision O.

LIMITATIONS: Limits of quantification are 0.01 ppm each compound for corn grain and crude oil samples and 0.02 ppm each compound for corn forage, fodder, and starch samples.

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BY:

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SUMMARY: Fipronil and its metabolites are extracted from corn material using acetonitrile. After clean-up by partition with hexane, fipronil and its metabolites are extracted by liquid-liquid partition with dichloromethane. The residues are then cleaned using silica gel and charcoal column prior to quantification by gas chromatography with electron capture detection.

I. **SUMMARY/INTRODUCTION**

Fipronil and its metabolites are extracted from corn material using acetonitrile. After clean-up by partition with hexane, fipronil and its metabolites are extracted by liquid-liquid partition with dichloromethane. The residues are then cleaned using silica gel and charcoal column prior to quantification by gas chromatography with electron capture detection.

The method has been validated for fipronil and its metabolites in field corn raw agricultural commodities: grain, forage, and fodder; and processed fractions: crude oil and starch. The method validation was conducted under Rhône-Poulenc Ag Company Study Number EC-93-236. Limits of quantification of this method for fipronil or its metabolites in corn grain and crude oil are 0.01 ppm for each compound. In corn forage, fodder, and starch, the limits of quantification are 0.02 ppm for each compound. Method detection limits are varied from 0.001 ppm to 0.008 ppm of each compound in field corn substrates and processed fractions.
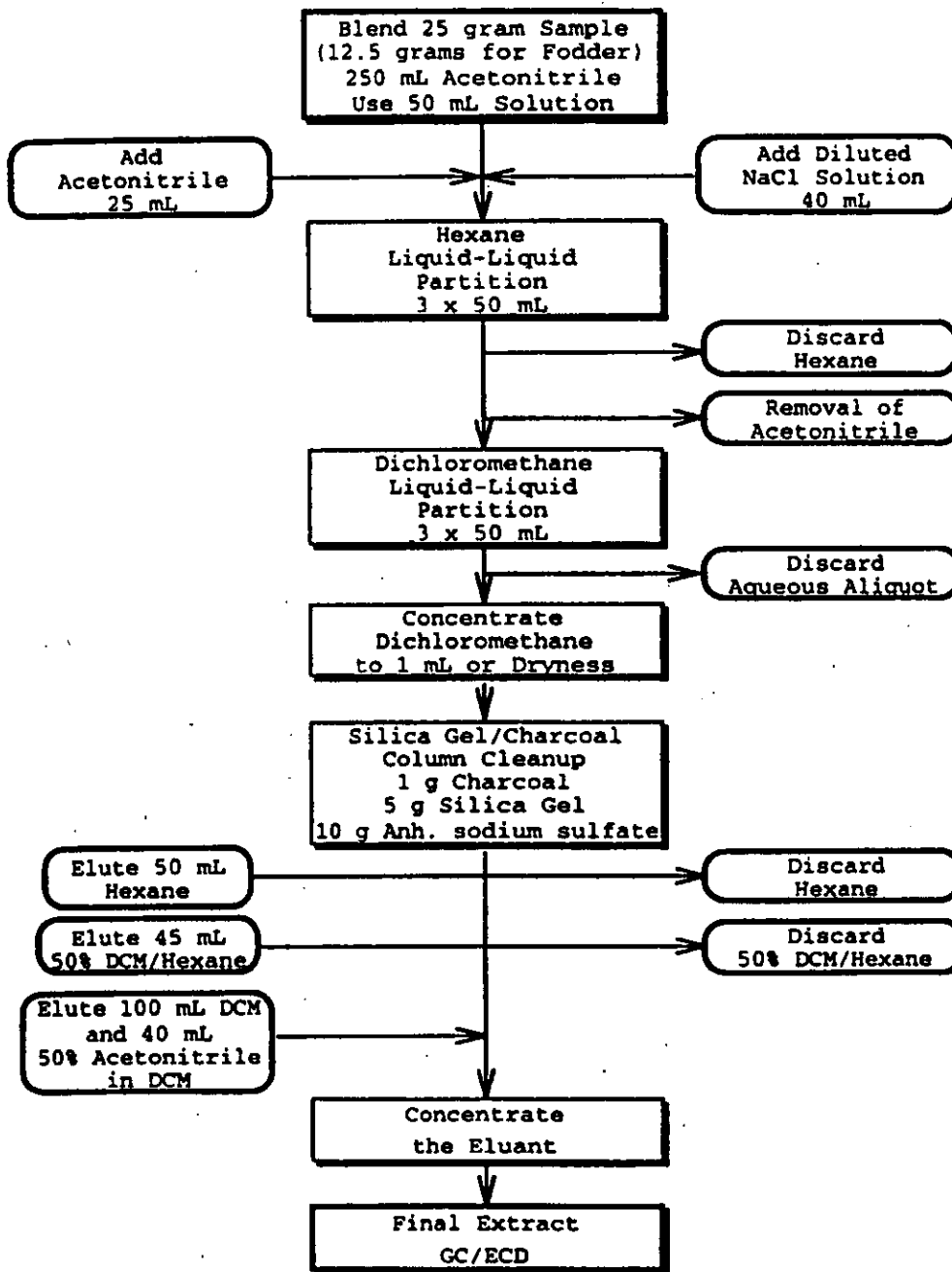
The parent fipronil, 5-amino-1-(2,6-dichloro-4-trifluoromethylphenyl)-3-cyano-4-trifluoromethanesulphinyl-pyrazole (MB 46030), is an insecticide developed by the Rhône-Poulenc Ag Company. Results in a metabolism study (EC-91-149) on corn have shown that the principal residues to be expected in corn raw agricultural commodities are fipronil (MB 46030), the active ingredient, and various metabolites. The metabolic pattern is shown in the table below.

Commodity	Fipronil and Metabolites present
Grain	MB 46030
Forage	MB 46030 and RPA 105048
Fodder	MB 46030, MB 45950, MB 46136, RPA 105048, and RPA 200766

Although only parent was identified in the grain, and parent and metabolite RPA 105048 in forage, the method validated includes on all compounds in the matrices of interest (grain, forage, fodder, starch, and crude oil).

The method of analysis flow chart is shown below.

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                          ┌──────────────────────────┐
                          │ Blend 25 gram Sample     │
                          │ (12.5 grams for Fodder)  │
                          │ 250 mL Acetonitrile      │
                          │ Use 50 mL Solution       │
                          └──────────────────────────┘
   ┌──────────────┐                    │                    ┌──────────────────┐
   │ Add          │                    │                    │ Add Diluted      │
   │ Acetonitrile │───────────────►◄───┤◄───────────────────│ NaCl Solution    │
   │ 25 mL        │                    ▼                    │ 40 mL            │
   └──────────────┘         ┌──────────────────────┐        └──────────────────┘
                            │ Hexane               │
                            │ Liquid-Liquid        │
                            │ Partition            │
                            │ 3 x 50 mL            │
                            └──────────────────────┘
                                       │              ┌──────────────────┐
                                       │─────────────►│ Discard          │
                                       │              │ Hexane           │
                                       │              └──────────────────┘
                                       │              ┌──────────────────┐
                                       │─────────────►│ Removal of       │
                                       ▼              │ Acetonitrile     │
                            ┌──────────────────────┐  └──────────────────┘
                            │ Dichloromethane      │
                            │ Liquid-Liquid        │
                            │ Partition            │
                            │ 3 x 50 mL            │
                            └──────────────────────┘
                                       │              ┌──────────────────┐
                                       │─────────────►│ Discard          │
                                       ▼              │ Aqueous Aliquot  │
                            ┌──────────────────────┐  └──────────────────┘
                            │ Concentrate          │
                            │ Dichloromethane      │
                            │ to 1 mL or Dryness   │
                            └──────────────────────┘
                                       ▼
                            ┌──────────────────────┐
                            │ Silica Gel/Charcoal  │
                            │ Column Cleanup       │
                            │ 1 g Charcoal         │
                            │ 5 g Silica Gel       │
                            │10 g Anh. sodium sulfate│
                            └──────────────────────┘
   ┌──────────────┐                    │              ┌──────────────────┐
   │ Elute 50 mL  │────────────────────┼─────────────►│ Discard          │
   │ Hexane       │                    │              │ Hexane           │
   └──────────────┘                    │              └──────────────────┘
   ┌──────────────┐                    │              ┌──────────────────┐
   │ Elute 45 mL  │────────────────────┼─────────────►│ Discard          │
   │ 50% DCM/Hexane│                   │              │ 50% DCM/Hexane   │
   └──────────────┘                    │              └──────────────────┘
   ┌──────────────┐                    │
   │ Elute 100 mL DCM│                 │
   │ and 40 mL    │───────────────────►│
   │ 50% Acetonitrile│                 ▼
   │ in DCM       │        ┌──────────────────────┐
   └──────────────┘        │ Concentrate          │
                           │ the Eluant           │
                           └──────────────────────┘
                                       ▼
                           ┌──────────────────────┐
                           │ Final Extract        │
                           │ GC/ECD               │
                           └──────────────────────┘
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The method consists of the extraction by blending the ground sample with acetonitrile using a homogenizer; filtration of the sample for removal of solids; the addition of a dilute sodium chloride solution to the acetonitrile aliquot and liquid-liquid partition cleanup using hexane; removal of the acetonitrile from the aqueous solution using TurboVap-evaporator; extraction of the aqueous solution with dichloromethane; concentration of the dichloromethane; column cleanup using silica gel and charcoal column with hexane and 50% dichloromethane in hexane; elution of analytes from the column with dichloromethane and 50% acetonitrile in dichloromethane; concentration of the combined extract; dilution of the extract with acetonitrile; and final quantification by gas chromatography using electron capture detection.

II.   **STANDARDS/EQUIPMENT/REAGENTS/SOLUTIONS/METHOD**

    A. ANALYTICAL STANDARDS

MB 46030 (fipronil):	5-amino-1-(2,6-dichloro-4-trifluoromethyl-phenyl)-3-cyano-4-trifluoromethanesulphinyl-pyrazole
MB 45950:	5-amino-1-(2,6-dichloro-4-trifluoromethylphenyl)-3-cyano-4-trifluoromethyl-thio-pyrazole
MB 46136:	5-amino-1-(2,6-dichloro-4-trifluoromethylphenyl)-3-cyano-4-trifluoromethylsulphonylpyrazole
RPA 105048:	1-(2,6-dichloro-4-trifluoromethylphenyl)-3-amido-5-amino-4-trifluoromethylpyrazole
RPA 200766:	5-amino-3-carbamoyl-1-(2,6-dichloro-4-trifluoro-methylphenyl)-4-trifluoromethylsulphinylpyrazole

ANALYTICAL STANDARD STRUCTURES

O
$\|$
F_3CS CN

H_2N N

Cl Cl

CF_3

MB 46030

F_3CS CN

H_2N N

Cl Cl

CF_3

MB 45950

O
$\|$
F_3C—S CN
$\|$
O

H_2N N

Cl Cl

CF_3

MB 46136

O
$\|$
F_3C CNH_2

H_2N N

Cl Cl

CF_3

RPA 105048

O O
$\|$ $\|$
F_3CS CNH_2

H_2N N

Cl Cl

CF_3
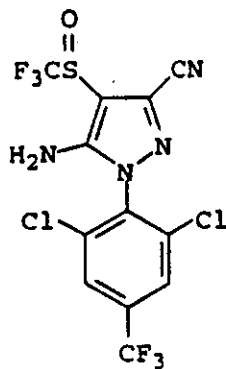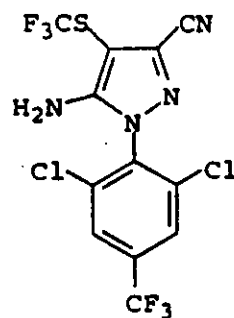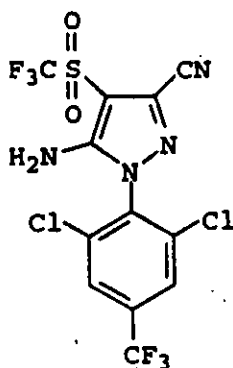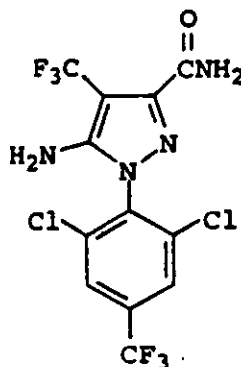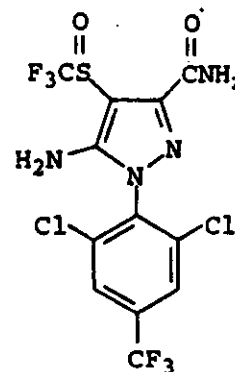
RPA 200766

B. EQUIPMENT

- Aluminum Crimp-top Seal with Teflon/silicone/Teflon septum, Cat. No. 200-148, Sun Brokers, Inc., or equivalent

- Analytical Balance, weigh accurately to ±0.1 mg, Mettler AK 160, or equivalent

- Top Load Balance, weigh accurately to ±1 mg, Mettler PE 2000, or equivalent

- Brinkmann Dispensette, 5-25 mL, or equivalent

- Buchner Funnel, 85 mm diameter, Coors Cat. 60242, or equivalent

- Chemical Trap, HP Part Nos. 05890-61260 and 19231-06790, Hewlett Packard Co., or equivalent

- Chromatographic Column, about 25 cm long, about 1.4 cm ID and about 250 mL round ball reservoir

- Concentrator Tube, 200 mL with 1 mL end point, Cat. No. ZW2038, Zymark, or equivalent

- Filter Paper, Whatmann Filter No. 2, 70 mm diameter, Cat. No. 1002070, or equivalent

- Gas Chromatograph, Hewlett Packard 5890 Series II or equivalent, equipped with ^{63}Ni electron capture detector model G1223A, G1224A, or equivalent

- Glass liner, 4 mm ID deactivated split-splitless Inlet sleeves (or glass liner), 6.5 mm x 78.5 mm, Cat. No. 20774, Restek Corporation, or equivalent

- Glasswool, Pyrex® fiber glass Sliver 8 micron, Cat. No. 3950

- Graduated cylinder, 250, 500, 1000 mL with ground glass joint stoppers, Fisher, or equivalent

- Glass jar, 200 mL

- Homogenizer, VirTis Cyclone Homogenizer, Part No. 278127, 20 mm shaft, Part No. 225227, and 20 mm macro generator head, Part No. 225219, VirTis Company, or equivalent

- Separatory Funnel, 250 mL

- Suction Adapter, side hose connection, 3 cm drip tip and ground glass joint $\bar{s}$ 27 adapter at the bottom, Southeastern Lab Apparatus, Inc. or equivalent

- TurboVap II Concentration Workstation, Cat. No. ZW8002, Zymark, or equivalent

- Volumetric Flask, appropriate sizes of A class volumetric flask

- Volumetric Pipet, appropriate sizes of A class volumetric pipet

C. REAGENTS

- Activated carbon (Charcoal), Darco®, 20-40 mesh, granular, Aldrich, Cat. No. 24,226-8, or equivalent

- Anhydrous Sodium sulfate, Granular Analytical Reagent, Malinckrodt, Cat. No. 8024, or equivalent

- Silica Gel 60, 63-200 microns, ICN Biomedicals, Cat. No. 04663, or equivalent

- Sodium Chloride, Crystal, Fisher S271-500, or equivalent

- Acetonitrile UV, High Purity Solvent, Burdick & Jackson, CAS 75-05-8, Cat. No. 015-4, or equivalent

- Dichloromethane, Burdick & Jackson ChromPure HPLC Solvent, CAS 75-09-2, Cat. No. CP80175-4, or equivalent

- Hexane, Burdick & Jackson High Purity Solvent, CAS 110-54-3, Cat No. 216-4, or equivalent

- Water, purified deionized water

- 0.5% Sodium chloride solution, dissolve 5 grams of sodium chloride in 1 liter of deionized water

- 50% Dichloromethane in Hexane, mix 1:1 dichloromethane in hexane

- 50% acetonitrile in dichloromethane, mix 1:1 acetonitrile in dichloromethane

D. STANDARD SOLUTION PREPARATION

Standard Solution Preparation of Each Standard

a. Using an analytical balance, weigh the analytical standard accurately (±0.1 mg) in a 100 mL volumetric flask to obtain target weight of 50 mg after standard purity correction. Dissolve in acetonitrile, dilute and mix well before bringing to

the 100 mL mark. Mix well. This solution contains 500 µg/mL of the standard in acetonitrile. Other concentrations may be used as required.

b. Make two serial 10 to 100 dilutions of the 500 µg/mL solution with acetonitrile in 100 mL volumetric flasks to give solutions containing 50 µg/mL and 5 µg/mL. Other dilutions may be used as required.

Standard Mixture Preparation

a. Standard Mixture for Spike

Using a volumetric pipet, pipet 5.0 mL of each 5 µg/mL standard solution into a single 100 mL volumetric flask and dilute with acetonitrile to 100 mL. Mix well. This standard mixture will contain 0.25 µg/mL of each standard. The 0.25 µg/mL solution may be used for sample spiking. Other dilutions may be used as required.

b. Standard Mixture for GC

Using a volumetric pipet, pipet 1.0 mL, 2.0 mL, 4.0 mL, 6.0 mL and 8.0 mL of the 0.25 µg/mL standard mixture into five 100 mL volumetric flasks and dilute with acetonitrile to 100 mL to give standard solutions containing 2.5 ng/mL, 5 ng/mL, 10 ng/mL, 15 ng/mL, and 20 ng/mL, respectively. These standard solutions will be used for gas chromatography standards. Other dilutions may be used as required.

E. ANALYTICAL PROCEDURE

1. ACETONITRILE EXTRACTION

1.1 Weigh 25.0 grams of sample (12.5 grams of fodder) into a 200-mL jar. This amount will be defined as the original total weight. Fortification sample for recovery determination and reference spike solution preparation should be done at this point.

Recovery Fortification

A 0.01 ppm (µg/g) spike would require the addition of 1.0 mL (by volumetric pipet) of the 0.25 µg/mL standard mixture solution into a 25.0 gram sample. Other spike levels may be used as required.

Reference Spike Solution Preparation:

A reference spike solution will be prepared at each recovery fortification level of the UTC sample. For a given fortification level, pipet an equal volume of the standard mixture, used in the recovery fortication of the UTC sample, into an appropriately sized volumetric flask. Dilute the reference spike to the volume mark with acetonitrile. The reference spike must be diluted to the same final concentration as the corresponding recovery spike (fortified UTC). The reference spike solution will be injected along with the sample set. The results of fipronil and its metabolites in the reference spike solution will be used in percent recovery calculations.

1.2 Add approximately 100 mL of acetonitrile to the 200-mL jar and homogenize at appropriate speed for approximately 5 minutes.

For all samples except crude oil and refined oil samples:

1.3 Filter the homogenate into a 250-mL graduated cylinder using a Buchner funnel, #2 filter paper, and a suction adapter under vacuum.

1.4 Retrieve at least 90 % the solid sample from the Buchner funnel, transfer back to the 200-mL jar and repeat the acetonitrile extraction steps 1.2 to 1.3.

1.5 Rinse the jar at least three times with approximately 10 mL portions of acetonitrile and repeat filtration step 1.3. Dilute the acetonitrile extract with additional acetonitrile to 250 mL total volume. Mix well before proceeding to step 2.1.

For crude oil and refined oil samples:

1.3 Transfer the homogenate into a 250-mL separatory funnel. Let the layers of the homogenate in the 250-mL separatory funnel separate, approximately 15 minutes. Transfer the bottom layer of the homogenate containing the oil portion and any middle layer back to the blending jar. Transfer the top layer of the homogenate into a cleaned 250-mL graduated cylinder.

1.4 Repeat steps 1.2 to 1.3. Discard the bottom layer and any middle layer of the homogenate containing the oil portion. Do not transfer back to the blending jar.

1.5 Rinse the blending jar with approximately 20 mL acetonitrile, transfer the rinsate into the 250-mL separatory funnel, and mix/swirl well. Let the layers of the homogenate in the 250-mL separatory funnel separate. Discard the bottom layer containing the oil portion, if any. Transfer/combine the top layer of the homogenate into the 250-mL graduated cylinder. Dilute the acetonitrile extract with acetonitrile to 250 mL. Mix well before proceeding to step 2.1.

   Notes: 1. This 250 mL acetonitrile extract contains material from the original total weight of the sample.

   2. Overnight stopping is acceptable. If so, the acetonitrile extract must be stored overnight in freezer.

2. HEXANE CLEANUP

2.1 Pipet a 50 mL portion of the acetonitrile extract obtained in step 1.5 and transfer to a 250-mL separatory funnel. This 50 mL acetonitrile extract contains material from the equivalent of one-fifth of the original total weight of the sample. Pipet a 25 mL portion of acetonitrile solvent into the acetonitrile extract in the 250-mL separatory funnel.

2.2 Add approximately 40 mL of a solution of 0.5% sodium chloride in deionized water to the separatory funnel.

2.3 Add approximately 50 mL hexane into the separatory funnel. Shake vigorously for approximately 30 seconds and let the layers separate.

2.4 Drain and collect the aqueous-acetonitrile layer. Discard the hexane aliquot. Transfer the aqueous extract back to the separatory funnel.

2.5 Repeat extraction steps 2.3 to 2.4. Repeat extraction step 2.3 one additional time.

2.6 Transfer the aqueous-acetonitrile extract into a 200-mL TurboVap concentrator tube. Discard the hexane aliquot.

2.7 Evaporate the aqueous-acetonitrile extract (using TurboVap concentrator) to remove the acetonitrile.

Notes: 1. A 35 mL mark on the 200-mL concentrator tube will assure the completion in removal of acetonitrile.

2. TurboVap bath temperature is approximately 55 °C.

3. DICHLOROMETHANE EXTRACTION

3.1 Transfer the aqueous extract to a clean 250-mL separatory funnel.

3.2 Rinse the 200-mL concentrator tube with 50 mL dichloromethane, transfer to the separatory funnel, shake vigorously for approximately 30 seconds and let the layers separate. Drain the dichloromethane layer into a clean 200-mL concentrator tube.

3.3 Repeat extraction step 3.2 two more times and combine the dichloromethane extracts. Discard the aqueous aliquot.

3.4 Evaporate the dichloromethane extract (using TurboVap concentrator) to about 1 mL or dryness.

Note: TurboVap bath temperature is approximately 35 °C.

4. SILICA GEL/CHARCOAL COLUMN CLEANUP

4.1 Prepare a silica gel/charcoal column; place a small amount of glasswool at the bottom of the column, add 1.0 gram of charcoal into the column, wash the charcoal with a 25 mL portion of acetonitrile followed by a 25 mL portion of dichloromethane, place a small amount of glasswool on top of charcoal, add about 35 mL of dichloromethane into the charcoal column, slowly add 5.0 grams silica gel using a clean funnel, slowly elute the dichloromethane and rinse several times with 1-2 mL of dichloromethane using micro pipette to let the silica gel pack into the column, elute until the level of dichloromethane is just above the silica gel, place a small amount of glasswool on top of silica gel, add 5 to 10 mL of

hexane into the column, add approximately 10 grams anhydrous sodium sulfate into the column using a clean funnel, wash the column with 25 mL of hexane and elute until the level of hexane is at the top of the sodium sulfate. Discard the eluant.

Notes: 1. Heat silica gel to approximately 110 °C overnight and then cool to room temperature before immediate use. The silica gel may be stored in an oven at approximately 110 °C.

2. Wash a 100 gram portion of charcoal in a Buchner funnel with filter paper with 300 to 400 mL of acetonitrile followed by 300 mL of dichloromethane. Dry by aspirating dichloromethane from the charcoal in the Buchner funnel. The clean charcoal may be stored in an oven at approximately 110 °C.

3. Rate of elution in step 4.1 is approximately 3 to 4 drops per second.

4.2 Adjust the dichloromethane extract volume in the 200-mL concentrator tube from step 3.4 until the 1 mL volume mark is reached. Quantitatively transfer the 1 mL dichloromethane extract from the 200-mL concentrator tube from step 3.4 (using a glass disposable micropipet) to the top of the sodium sulfate of the column.

4.3 Carefully rinse the 200-mL concentrator tube twice with approximately 1 mL portion of dichloromethane and repeat transfer step 4.2.

4.4 Elute the dichloromethane extract until the level at the top of the sodium sulfate. Discard the eluant.

Note: Rate of elution in step 4.4 and the remaining steps will be approximately 2 to 3 drops per second.

4.5 Rinse the 200-mL concentrator tube with 50 mL of hexane and transfer to the column. Elute until the level of the hexane is at the top of the sodium sulfate and discard the eluant.

4.6 Rinse the 200-mL concentrator tube with 45 mL of 50% dichloromethane in hexane and transfer to the column.

Elute until the level of the mobile phase is at the top of the sodium sulfate and discard the eluant.

4.7 Rinse the 200-mL concentrator tube with 100 mL of dichloromethane, transfer to the column and elute until the level of the mobile phase is at the top of the sodium sulfate. Collect the eluant in a clean 200-mL concentrator tube.

4.8 Rinse the former 200-mL concentrator tube with 40 mL of 50% acetonitrile in dichloromethane, transfer to the column, elute until the level of the mobile phase is at the top of the sodium sulfate. Collect and combine with the eluant from step 4.7.

4.9 Evaporate the combined eluant to about 1 mL and then quantitatively transfer/rinse the 200-mL concentrator tube with acetonitrile into a 5 mL-volumetric flask until the volume mark is reached. Other appropriate final volume may be obtained with the higher fortified level.

   Notes: 1. This 5 mL final extract contains material from the equivalent of one-fifth of the original total weight of the sample.

   2. TurboVap bath temperature is approximately 35 °C.

5. INSTRUMENTATION

5.1 Gas chromatograph system consists of a Hewlett-Packard 5890 Series II GC or equivalent equipped with an autosampler (H/P model #7673 or equivalent), sampler control (H/P model #18594B or equivalent), integrator (H/P model #3396A or equivalent), and a split/splitless injector. A HP integrator will be used to control the start/stop of system. The chromatograms and data from the HP integrator will be used as data verification.

5.2 An electron capture detector will be used for GC detection (ECD: H/P models G1223A, G1224A, or equivalent).

5.3 A Waters® 860 Data Capture system or equivalent will be utilized for chromatographic data acquisition.

5.4 Analyze a 1.0 µL portion of each standard mixture and each final extract sample, by gas chromatography using electron capture detection.

5.5 Injector - Capillary split/splitless inlet system, using a 0.5 minute splitless injection mode

5.6 Column - FSOT HP-1 column with 12.5 meter long, 0.2 mm ID, and 0.33 µm film thickness, Hewlett Packard Company, HP Part No. 19091-60312, or equivalent

5.7 GC Conditions:

Injector temperature	250 °C
Detector temperature	300 °C
Total flow	Helium - about 75 cc/min
Carrier gas/septum purge	Helium - about 0.6 cc/min for column flow at initial temperature (approximately 9 psig, constant head pressure), and about 0.5 to 1 cc/min for septum purge
Detector make-up gas	5% Methane in argon at about 45 cc/min in auxiliary gas and anode purge (approximately 46 psig)
Initial temp	60 °C
Initial hold	0 min
First temp rate	20 °C/min
First final temp	120 °C
First final hold	0 min
Second temp rate	2.5 °C/min
Second final temp	240 °C
Second final hold	0 min
Third temp rate	20 °C/min
Third final temp	275 °C
Third final hold	20 min
Equilibrium time	8 min

Notes: 1. These GC parameters are guidelines and can be optimized for the chromatograph and column actually used. Any minor modifications will be recorded in the method of the data acquisition.

(Notes are continued to next page)

2. Retention Time (R_t)

MB 45950:	R_t is approximately 32 minutes.
MB 46030:	R_t is approximately 33 minutes.
RPA 105048:	R_t is approximately 37 minutes.
MB 46136:	R_t is approximately 38 minutes.
RPA 200766:	R_t is approximately 44 minutes.

The retention time of each compound will depend on the GC conditions, the chromatograph and the column actually used.

3. New column will be conditioned at approximately 250°C about 4 hours before installing to ECD detector and continued conditioning overnight before use.

4. The column may be reconditioned at approximately 250°C before use.

6. CALCULATIONS:

Fipronil and its metabolites, whether present as residues or fortifications, will be quantified by comparison to standard curves obtained from data linear regression (concentration ng/mL injected vs. peak area).

The reference spike solution prepared for each analytical set will also be quantified by the same set of calibration curve. The results of the reference spike solution, µg found, will be used in recovery calculations.

$$ppb \ (ng/g) \quad = \quad \frac{(H - A)/B}{SA} \times SF$$

where,

H	=	Test sample peak area
A	=	Intercept from regression analysis of standards
B	=	Slope from regression analysis of standards
SF	=	Scale factor (dilution factor, mL)
SA	=	The equivalent sample amount based upon amount of acetonitrile extract solution used (grams)

Percent Recovery $= \dfrac{\mu g \text{ Found in Spike} - \mu g \text{ Found in UTC}}{\mu g \text{ Found in reference spike}} \times 100$

where,

μg found in spike $=$ ng/g found in spike x total sample weight / 10·

μg found in UTC $=$ ng/g found in UTC x total sample weight / 1000

μg found in reference spike $=$ ng found in reference spike / 1000.

F. INTERFERENCE

Forty five of the registered pesticides listed in the 40 CFR, including those most heavily used in corn field applications, were analyzed for fipronil and its metabolite interference testing. It was concluded that there are no significant interferences of other registered pesticides in this method of analysis for fipronil and its metabolites.

G. CONFIRMATORY TECHNIQUES

A HP-5 capillary column (12.5 meters, 0.25 mm, 0.33 μm film thickness) was used in method confirmation for fipronil and its metabolites. The analysis showed that the HP-5 column can be used only qualitatively for method confirmation.

A gas chromatography / mass spectrometry (GC/MS) with single ion recording (SIR) mode was used qualitatively as an additional technique for method confirmation.

H. TIME REQUIRED FOR ANALYSIS

A set of four to eight samples can be prepared for GC analysis within a twelve hour time period. The samples can be carried through the acetonitrile extraction (through step 1.5) in a 4 hour time period. Hexane cleanup, column chromatography cleanup, and extract preparation for GC analysis can be carried at in an 8 hour time period.

I. POTENTIAL PROBLEMS OR COMMENTS

1. Acetonitrile UV high purity solvent grade (B&J) is recommended for this method.

2. In addition to the moisture traps normally installed, in-line chemical traps are recommended for both the carrier gas and make-up gas lines to remove any bleed of contaminants from the flow controllers (see equipment section).

3. A deactivated inlet sleeve is recommended (see equipment section).

4. A new column should be conditioned to approximately 250 °C about 4 hours before installing into the ECD detector and continued conditioning at approximately 250 °C overnight before use. This is necessary in order to keep the ECD detector clean and to stabilize the baseline of the chromatogram when temperature programming is used with ECD detection.

5. The incomplete removal of acetonitrile, in step 2.7 under the hexane cleanup section, will result in lower recoveries of MB 45950 and MB 46030. Evaporation of the aqueous-acetonitrile extract to about 35 mL is recommended.

6. Due to a crop matrix enhancement, peak responses of the MB 46136, RPA 105048, and RPA 200766 metabolites in the samples are higher than in the standard solutions. Therefore, high recoveries of the MB 46136, RPA 105048, and RPA 200766 metabolites are observed after injecting several sets of samples. This problem is usually solved by replacing the inlet sleeve, gold seal and washer supporting the inlet sleeve beneath the injector (HP 5890 GC), and/or removing about 0.5 meter of column inlet.

III. RESULTS AND DISCUSSION

A. METHOD DETECTION LIMIT (MDL) AND LIMIT OF QUANTIFICATION (LOQ)

The method detection limit (MDL) is defined as the minimum concentration of the analyte that can be measured and reported with 99% confidence that the analyte concentration is greater than zero. The determination of the MDL of each compound was obtained by the summation of the average residue of the UTC samples and three times the sample standard deviation of the estimated MDL spiked samples;

$$MDL_i = \bar{x}_i(UTC) + 3 \times S.D._i(spike), \text{ where } i \text{ is parent or a metabolite.}$$

The limit of quantification (LOQ) is defined as the level of analyte in the substrates above which quantitative results are obtained with a specified degree of confidence. Confidence in the apparent results increase as the analyte signal increases above the limit of detection. The determination of the LOQ of each compound was obtained by the summation of the average residue of the UTC samples and ten times the sample standard deviation of the estimated MDL spiked samples;

$$LOQ_i = \bar{x}_i(UTC) + 10 \times S.D._i(spike), \text{ where } i \text{ is parent or a metabolite.}$$

In MDL and LOQ determinations, five UTC samples and seven UTC fortified at level of estimated MDL, 5 ppb (0.005 ppm), each of the fipronil and its metabolites were analyzed on two different days. This estimated MDL of 5 ppb was selected from the method development prior to the method validation. TABLES I to V tabulate the results of the MDL and LOQ experiments for the RAC field corn grain, forage, fodder, and corn processed fraction starch, and crude oil, respectively. The MDL and LOQ results are summarized in table below. A "selected" LOQ was established by rounding the highest LOQ of the fipronil or its metabolites.

MDL/LOQ Substrate		MB46030	MB45950	MB46136	RPA105048	RPA200766
		ppm (µg/g)				
MDL	Grain	0.002	0.002	0.003	0.003	0.003
LOQ		0.006	0.006	0.007	0.007	0.008
LOQ	(selected)	0.010	0.010	0.010	0.010	0.010
MDL	Forage	0.001	0.003	0.005	0.002	0.003
LOQ		0.004	0.008	0.015	0.004	0.006
LOQ	(selected)	0.020	0.020	0.020	0.020	0.020
MDL	Fodder	0.003	0.007	0.005	0.008	0.006
LOQ		0.010	0.013	0.016	0.019	0.017
LOQ	(selected)	0.020	0.020	0.020	0.020	0.020
MDL	Starch	0.004	0.003	0.004	0.006	0.006
LOQ		0.010	0.009	0.010	0.018	0.018
LOQ	(selected)	0.020	0.020	0.020	0.020	0.020
MDL	Crude Oil	0.002	0.002	0.002	0.002	0.004
LOQ		0.006	0.007	0.006	0.005	0.006
LOQ	(selected)	0.010	0.010	0.010	0.010	0.010

B. PRECISION AND ACCURACY

In precision and accuracy determinations, two UTC samples, five fortified samples at a selected LOQ, and five fortified samples at a level 5 times of selected LOQ were analyzed for fipronil and its metabolites on two different days. The LOQ's selected are 0.01 ppm each of fipronil and its metabolites in grain and crude oil, and 0.02 ppm each of fipronil and its metabolites in forage, fodder, and starch. TABLES I to V tabulate the results of the precision and accuracy experiments for the RAC field corn grain, forage, fodder, and corn processed fraction starch, and crude oil, respectively. The statistical data include ranges of recoveries, means of recoveries (accuracy of the method), standard deviations, and percent relative standard deviations (precision of the method). In the validation data, the mean of recovery of each analyte was within 70% to 120%, except for the following: MB 45950 in grain (67% and 69%); MB 45950 in forage (61% and 57%); MB 45950 in fodder (58%); RPA 105048 in fodder (127%); MB 45950 in starch (67% and 69%); MB 45950 in crude oil (67%); and RPA 105048 in crude oil (127%).

The overall precision and accuracy results of all validated matrices
are summarized in table below.

	MB46030	MB45950	MB46136	RPA105048	RPA200766
Min % Recovery	62	39	66	72	64
Max % Recovery	115	101	130	144	130
Mean	81	65	99	108	100
S.D.	11	12	14	18	15
%RSD	13	19	14	17	15

From the overall precision and accuracy results, the proposed
recovery ranges for the method are 60% to 120% for MB 46030;
50% to 100% for MB 45950; 70% to 130% for MD 46136, 70% to 140% for
RPA 105048; and 70% to 130% for RPA 200766.

C. EXTRACTION EFFICIENCY

In extraction efficiency testing, the analyses showed acceptable
recovery results of fipronil and its metabolites in aged corn grain
samples.

D. RUGGEDNESS TESTING

1. In reagent blank test, the analyses showed that there is no
significant background of apparent fipronil and its metabolites.

2. RotaVap evaporator was used instead of TurboVap evaporator for the
ruggedness testing in the evaporation steps of the method. The
analyses showed that RotaVap evaporator can be used instead of
TurboVap evaporator in the method. If a RotaVap evaporator is
used, a Buchi Rotavapor R110 (or equivalent) and a Kontes
evaporator trap, Article No. 570210-0124, 100 mL capacity (or
equivalent) are recommended. Multiple rinsing of the flask as in
steps 2.6, 3.2, and 3.2 to 4.9 is required.

3. A ruggedness test, in evaporation of the dichloromethane to
dryness instead of to 1 mL in step 3.4 of the Dichloromethane
Extraction Section, showed no losses of the fipronil and its
metabolites.

E. LIMITATIONS

This method has only been validated for the corn field substrates in
the range of 0.01 to 0.05 ppm of each compound for fipronil and its
metabolites in grain and crude oil samples and 0.02 to 0.1 ppm of

each compound for fipronil and its metabolites in forage, fodder, and starch samples. The range of linearity testing was performed only in corn grain. The analyses showed that the method is capable of extracting the residues with the linearity range up to 1 ppm each fipronil and its metabolites in grain substrate.

IV. CONCLUSION

This method of analysis has been validated for fipronil and its metabolites in field corn grain, forage, fodder, starch, and crude oil. This method is capable of quantifying each fipronil or its metabolites at 0.01 ppm for grain and crude oil, and at 0.02 ppm for forage, fodder, starch. The validation data of fodder should suffice for silage, crude oil data should suffice for refined oil, and grain data should suffice for the processed fractions; grits, meal, flour, and grain dust because of the similarity of the respective matrices.

V. NOTE

The following minor method modifications were made during the storage stability analysis (Protocol Amendment #1, 15 December 1993, Study No. EC-93-245) and prior to the 1993 corn RAC analysis (Protocol Amendment #2, 6 January 1994, Study No. US93V01R):

At the end of the step 2.1 of the method of analysis, an additional 25 mL portion of acetonitrile solvent is added to the 50 mL of the acetonitrile extract. This modification increases the organic solvent strength of the aqueous acetonitrile solution prior to the hexane partition clean-up step. This additional solvent improves the recovery results of MB 45950 and MB 46030. From the recovery results of the 1993 RAC field corn sample analyses (for grain, forage, fodder, and silage) in Study No. US93V01R (observed recovery ranges: 68% to 124% for MB 46030; 69% to 109% for MB 45950; 82% to 130% for MB 46136, 78% to 139% for RPA 105048; and 87% to 136% for RPA 200766), new expected recovery ranges are 70% to 130% for MB 46030; 70% to 120% for MB 45950; 70% to 130% for MB 46136, 80% to 140% for RPA 105048; and 80% to 140% for RPA 200766.

In the calculation section of the method of analysis, peak areas were used instead of peak heights. This modification was made to resolve the peak-height enhancement problem in the method of analysis.

Some minor changes were also made to the wording for clarification purposes.

Data for MDL, LOQ, precision, accuracry, ruggedness, and interferences were colected for this Study EC-93-236 using the unmodified method.

VI. REFERENCES: RPAC Study Number: EC-93-236 and RPAC Lab Notebook: EC-50-331

VII. TABLES

Results of the MDL, LOQ, the precision and accuracy experiments and summarized statistical data were shown in TABLES I to IV.

TABLE I. SUMMARY OF THE ANALYTICAL METHOD VALIDATION RESULTS OF
FIPRONIL AND ITS METABOLITES IN CORN GRAIN

			MB46030	MB45950	MB46136	RPA105048	RPA200766
					ppm		
Method Detection Limit (MDL)			0.002	0.002	0.003	0.003	0.003
Limit of Quantitation (LOQ)			0.006	0.006	0.007	0.007	0.008
LOQ (selected)			0.010	0.010	0.010	0.010	0.010
	Spike Level				Percent Recovery		
Spike 1	10 ppb	day 1	97	60	101	121	120
Spike 2	10 ppb	day 1	88	65	95	103	99
Spike 3	10 ppb	day 1	91	56	93	105	105
Spike 6	10 ppb	day 2	86	76	113	139	120
Spike 7	10 ppb	day 2	85	78	120	130	115
Spike 4	50 ppb	day 1	84	65	89	93	84
Spike 5	50 ppb	day 1	76	52	73	78	73
Spike 8	50 ppb	day 2	85	79	106	110	104
Spike 9	50 ppb	day 2	77	80	107	95	87
Spike 10	50 ppb	day 2	67	70	100	82	80
Mean of 5 Recoveries (10ppb)			90	67	105	119	112
SD (of 5)			5	10	12	16	9
%RSD (10ppb)			5	15	11	13	8
Mean of 5 Recoveries (50ppb)			78	69	95	92	86
SD (of 5)			7	11	14	13	12
%RSD (50ppb)			9	16	15	14	13

TABLE II.   SUMMARY OF THE ANALYTICAL METHOD VALIDATION RESULTS OF
FIPRONIL AND ITS METABOLITES IN CORN FORAGE

			MB46030	MB45950	MB46136	RPA105048	RPA200766
					ppm		
Method Detection Limit (MDL)			0.001	0.003	0.005	0.002	0.003
Limit of Quantitation (LOQ)			0.004	0.008	0.015	0.004	0.006
LOQ (selected)			0.020	0.020	0.020	0.020	0.020
	Spike Level				Percent Recovery		
Spike 1	20 ppb	day 1	66	69	100	93	90
Spike 2	20 ppb	day 1	70	68	104	103	100
Spike 3	20 ppb	day 1	71	69	105	104	100
Spike 6	20 ppb	day 2	81	54	97	106	105
Spike 7	20 ppb	day 2	83	45	83	104	104
Spike 4	100 ppb	day 1	64	69	108	97	96
Spike 5	100 ppb	day 1	62	68	100	92	90
Spike 8	100 ppb	day 2	76	50	98	112	110
Spike 9	100 ppb	day 2	74	49	100	112	109
Spike 10	100 ppb	day 2	76	47	88	107	104
Mean of 5 Recoveries (20ppb)			74	61	98	102	100
SD (of 5)			7	11	9	5	6
%RSD (20ppb)			10	18	9	5	6
Mean of 5 Recoveries (100ppb)			70	57	99	104	102
SD (of 5)			7	11	7	9	8
%RSD (100ppb)			10	20	7	9	8

FIGURE I   TYPICAL GC CHROMATOGRAM OF THE FIPRONIL AND ITS METABOLITE
STANDARD MIXTURE (0.02 µg/mL EACH)
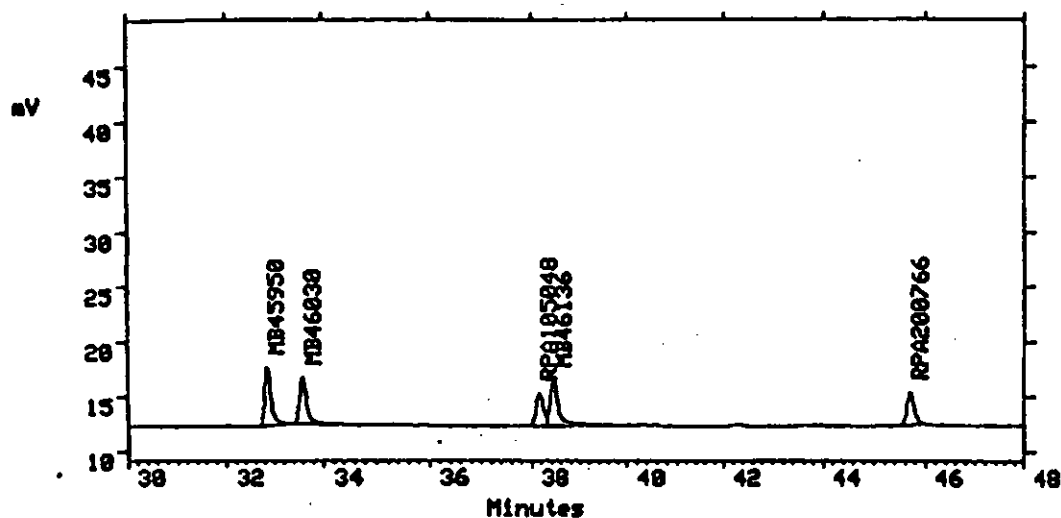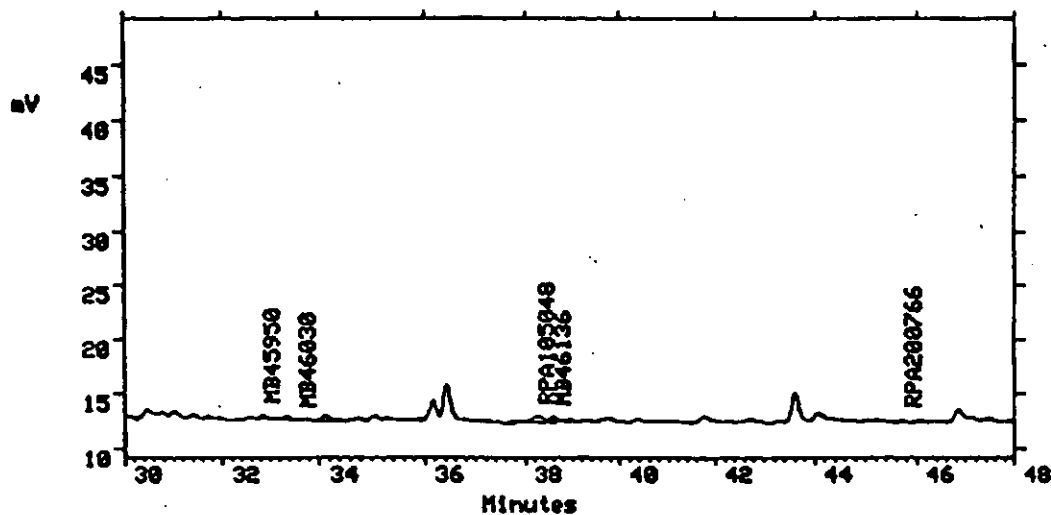
EC583381A45 Man Inj Vial   0 Inject 1 Ch 1

FIGURE II   TYPICAL GC CHROMATOGRAM OF THE UTC FODDER SAMPLE

RM5402UTC Man Inj Vial   0 Inject 1 Ch 1

FIGURE III   TYPICAL GC CHROMATOGRAM OF THE METHOD SPIKE FODDER SAMPLE
(0.02 PPM EACH COMPOUND)

RM5402MS Man Inj Vial ·0 Inject 1 Ch 1

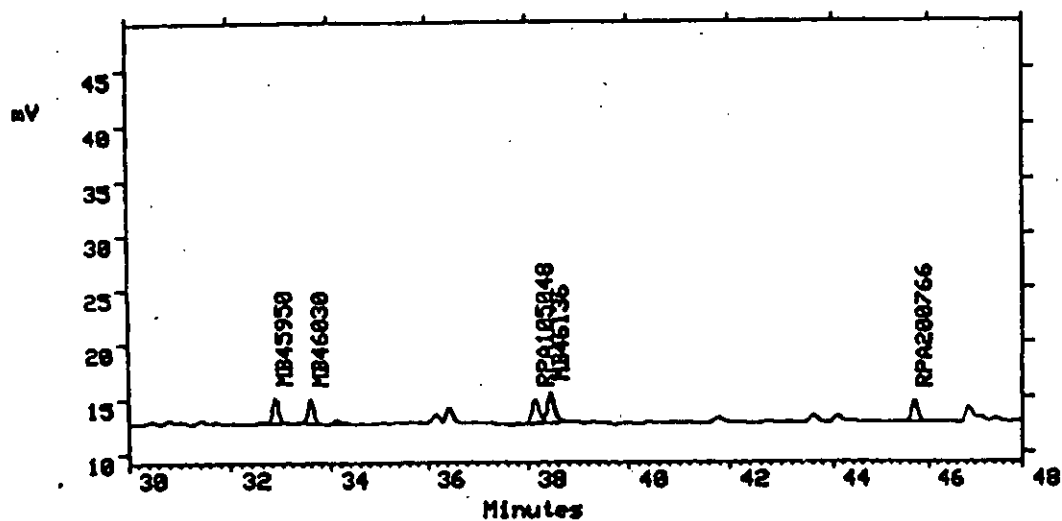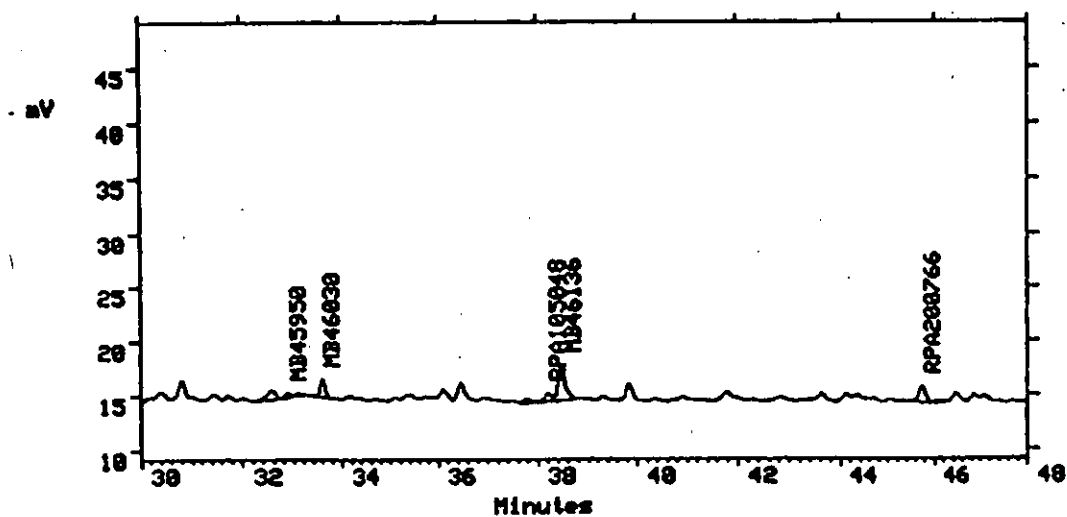FIGURE IV   TYPICAL GC CHROMATOGRAM OF THE TREATED FODDER SAMPLE

RM6345 Man Inj Vial 0 Inject 1 Ch 1

TABLE III. SUMMARY OF THE ANALYTICAL METHOD VALIDATION RESULTS OF
FIPRONIL AND ITS METABOLITES IN CORN FODDER

			MB46030	MB45950	MB46136	RPA105048	RPA200766
					ppm		
Method Detection Limit (MDL)			0.003	0.007	0.005	0.008	0.006
Limit of Quantitation (LOQ)			0.010	0.013	0.016	0.019	0.017
LOQ (selected)			0.020	0.020	0.020	0.020	0.020
	Spike Level				Percent Recovery		
Spike 1	20 ppb	day 1	85	66	114	144	122
Spike 2	20 ppb	day 1	84	59	113	140	116
Spike 3	20 ppb	day 1	76	49	88	127	104
Spike 6	20 ppb	day 2	74	57	87	114	102
Spike 7	20 ppb	day 2	71	58	69	112	99
Spike 4	100 ppb	day 1	82	85	108	114	101
Spike 5	100 ppb	day 1	77	53	93	109	95
Spike 8	100 ppb	day 2	89	63	105	131	119
Spike 9	100 ppb	day 2	85	52	89	122	112
Spike 10	100 ppb	day 2	79	39	66	122	109
Mean of 5 Recoveries (20ppb)			78	58	94	127	109
SD (of 5)			6	6	19	15	10
%RSD (20ppb)			8	11	20	12	9
Mean of 5 Recoveries (100ppb)			82	58	92	120	107
SD (of 5)			5	17	17	8	9
%RSD (100ppb)			6	30	18	7	9

TABLE IV.   SUMMARY OF THE ANALYTICAL METHOD VALIDATION RESULTS OF
FIPRONIL AND ITS METABOLITES IN CORN STARCH

			MB 46030	MB 45950	MB 46136	RPA 105048	RPA 200766
					ppm		
Method Detection Limit (MDL)			0.004	0.003	0.004	0.006	0.006
Limit of Quantitation (LOQ)			0.010	0.009	0.010	0.018	0.018
LOQ (selected)			0.020	0.020	0.020	0.020	0.020
	Spike Level				Percent Recovery		
Spike 1	20 ppb	day 1	76	54	109	93	86
Spike 2	20 ppb	day 1	79	65	104	109	89
Spike 3	20 ppb	day 1	89	68	101	85	76
Spike 6	20 ppb	day 2	63	60	68	72	64
Spike 7	20 ppb	day 2	89	86	117	123	107
Spike 4	100 ppb	day 1	67	67	99	79	77
Spike 5	100 ppb	day 1	70	68	104	79	77
Spike 8	100 ppb	day 2	73	67	77	80	76
Spike 9	100 ppb	day 2	83	78	95	92	91
Spike 10	100 ppb	day 2	70	66	86	82	78
Mean of 5 Recoveries (20ppb)			79	67	100	96	84
SD (of 5)			11	12	19	20	16
%RSD (20ppb)			13	18	19	21	19
Mean of 5 Recoveries (100ppb			72	69	92	82	80
SD (of 5)			6	5	11	6	6
%RSD (100ppb)			8	7	11	7	8

TABLE V.   SUMMARY OF THE ANALYTICAL METHOD VALIDATION RESULTS OF
FIPRONIL AND ITS METABOLITES IN CORN CRUDE OIL

			MB 46030	MB 45950	MB 46136	RPA 105048	RPA 200766
					ppm		
Method Detection Limit (MDL)			0.002	0.002	0.002	0.002	0.004
Limit of Quantitation (LOQ)			0.006	0.007	0.006	0.005	0.006
LOQ (selected)			0.010	0.010	0.010	0.010	0.010
	Spike Level				Percent Recovery		
Spike 1	10 ppb	day 1	82	76	102	99	96
Spike 2	10 ppb	day 1	84	76	92	97	90
Spike 3	10 ppb	day 1	94	74	107	117	117
Spike 6	10 ppb	day 2	90	63	98	116	100
Spike 7	10 ppb	day 2	88	45	85	130	106
Spike 4	50 ppb	day 1	86	77	117	114	108
Spike 5	50 ppb	day 1	93	84	121	122	116
Spike 8	50 ppb	day 2	91	57	111	131	119
Spike 9	50 ppb	day 2	104	73	111	126	118
Spike 10	50 ppb	day 2	115	101	130	141	130
Mean of 5 Recoveries (10ppb)			88	67	97	112	102
SD (of 5)			5	13	9	14	10
%RSD (10ppb)			5	20	9	12	10
Mean of 5 Recoveries (50ppb)			98	78	118	127	118
SD (of 5)			11	16	8	10	8
%RSD (50ppb)			12	21	7	8	7

VII. **FIGURES**

FIGURES I to IV showed the representative chromatograms showing a standard mixture at 0.01 µg/mL, an untreated control forage sample, an analytical method recovery sample (0.02 ppm), and a treated forage sample.

FIGURE I   TYPICAL GC CHROMATOGRAM OF THE FIPRONIL AND ITS METABOLITES STANDARDS

FIGURE II   TYPICAL GC CHROMATOGRAM OF THE UTC FODDER SAMPLE

FIGURE III   TYPICAL GC CHROMATOGRAM OF THE METHOD SPIKE FODDER SAMPLE

FIGURE IV   TYPICAL GC CHROMATOGRAM OF THE TREATED FODDER SAMPLE