

US EPA ARCHIVE DOCUMENT

**FINAL STUDY REPORT**

**STUDY TITLE**

Fipronil - Validation of Method of Analysis for Fipronil and  
its Metabolites in Animal Tissues

**DATA REQUIREMENT**

FIFRA Subdivision O, Part 171-4 (d),  
Residue Analytical Method - Animals

**STUDY DIRECTOR**

J. R. Hudson, Ph.D.  
Rhône-Poulenc Ag Company  
Research Triangle Park, NC

**STUDY DATES:**

Initiation: April 4, 1994  
Completion: September 9, 1994

**SPONSOR**

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Research Triangle Park, NC 27709

**PERFORMING LABORATORY / TESTING FACILITY**

Rhône-Poulenc Ag Company (RPAC)  
2 T.W. Alexander Drive  
Research Triangle Park, NC 27709

**RPAC STUDY NUMBER**

EC-94-258

**TOTAL NUMBER OF PAGES**

205

**STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS**

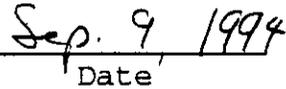
No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA §10 (d) (1) (A), (B), or (C).

Company: Rhône-Poulenc Ag Company

Company Agent:



Larry R. Hodges  
Registration Manager  
Rhône-Poulenc Ag Company



Date

**GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT**

This study, EC-94-258, was conducted in accordance with the Final Rule of the EPA FIFRA Good Laboratory Practice Standards (40 CFR 160; Federal Register 17 August 1989), with the following exceptions:

1. Characterization of chemicals and solutions obtained from Chem Service, EM Science, AccuStandard, and the US EPA Pesticides Repository, and which were used to test for method specificity, were not performed under FIFRA Good Laboratory Practice Standards.
2. Some raw data entries were not identified with initials and date and some corrections were not identified with initials, dates, and reasons for corrections (40 CFR 160.130(e)) in a timely manner. The exclusions were repetitive entry omissions and did not affect the study integrity.
3. Some notebook pages were not closed out with a diagonal line through unused portions of the pages in a timely manner. The exclusions were repetitive entry omissions and did not affect the study integrity.

Submitter:

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Sept 9, 1994  
Date

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Sept 9, 1994  
Date

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Rhône-Poulenc Ag Company

September 9, 1994  
Date

Quality Assurance Unit  
Statement of Inspections

The Analytical portion of this study, EC-94-258, was inspected by the Quality Assurance Unit according to Rhône-Poulenc Ag Company Standard Operating Procedures on the following dates:

March 24, 1994	August 25, 1994
May 10, 1994	August 25, 1994

The results of the inspections were reported to the Study Director on the following dates:

March 25, 1994	September 1, 1994
May 26, 1994	September 1, 1994

The results of the inspections were reported to Management on the following dates:

March 30, 1994	September 7, 1994
June 27, 1994	September 9, 1994

 9/9/94  
\_\_\_\_\_  
Anthony A. Gemma                      Date  
Compliance Manager

**AUTHENTICATION**

Study Number: EC-94-258

I hereby declare that this study was performed under my direction and that this report represents a true and accurate record of the results obtained.

Study Director:

J R Hudson

J. R. Hudson  
Principal Scientist I  
Environmental/Analytical Chemistry  
Rhône-Poulenc Ag Company

September 9, 1994

Date

Approval:

E J Breaux

E. J. Breaux  
Director  
Environmental/Analytical Chemistry  
Rhône-Poulenc Ag Company

Sept 9, 1994

Date

Samples of the test substances used in this study will be retained in the Rhône-Poulenc Ag Company Chemical Archives for as long as the quality of the material affords evaluation or for the duration of the registration. All test samples will be retained in frozen storage until the final report has been completed. All raw data, documentation, chromatograms, records, related correspondence, protocol and amendments, and the final report will be retained in the Rhône-Poulenc Ag Company Archives.

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APPENDIX A

STUDY PROTOCOL AND AMENDMENTS

(protocol pages 19-22 reduced to 78% of original for margin)

**METHOD VALIDATION STUDY PROTOCOL**

**STUDY NUMBER:** EC-94-258

**TITLE:** Fipronil - Validation of Method of Analysis for Fipronil and its Metabolites in Animal Tissues

**OBJECTIVE:** The objective of this study is to validate the method of analysis for residues of fipronil and its metabolites in animal tissues.

**JUSTIFICATION:** Method validation is required by the EPA for a method of analysis for fipronil and its metabolites in animal tissues (40 CFR 158.240, and Pesticide Assessment Guidelines, Subdivision O, 171-4(b)).

**TEST SUBSTANCE** Fipronil (MB46030) and Its Metabolites, MB 45950, and MB 46136. All test and reference substances will be supplied by Rhône-Poulenc Ag Company.

**TEST SYSTEM/  
SUBSTRATES:** Animal substrates as defined in Pesticide Assessment Guidelines, Subdivision O. Selected representative animal substrates to be validated are listed on page 3.

**SPONSOR/TESTING  
FACILITY:** Rhône-Poulenc Ag Company  
2 T.W. Alexander Drive  
Research Triangle Park, NC 27709

**STUDY DIRECTOR:** J. R. Hudson  
Food Safety and Residue Programs Group  
Rhône-Poulenc Ag Company  
(919)549-2501

**GLP COMPLIANCE:** This study will be conducted and reported in accordance with Good Laboratory Practice Regulations (FIFRA Final Rule, October 16, 1989). A statement of inspections and a statement of compliance will be included in the final study report.

Revisions in this protocol and the reasons for the changes are to be documented as protocol amendments, signed by the Study Director and Group Leader, and maintained with the protocol. All deviations from this protocol shall be documented in the raw data and authorized by the Study Director.

**PROPOSED STUDY EXPERIMENTAL SCHEDULE**

Proposed Study Experimental Start Date: March 30, 1994

Proposed Study Experimental Termination Date: April 30, 1994

**CONTENTS:**  
Part 1. Sample Information  
Part 2. Study Design  
Part 3. Analytical Information  
Part 4. Record Keeping / Quality Assurance

**STUDY DIRECTOR:** JRHudson DATE: April 4, 1994  
J. R. Hudson  
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**GROUP LEADER:** Timothy W Hunt DATE: 4 April 94  
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(919) 549-2504

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K. Prasad Rao  
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(919) 549-2302

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## PART 1 - SAMPLE INFORMATION

### 1.1 TEST SYSTEM IDENTIFICATION

#### Poultry Substrates:

1. Chicken breast muscle
2. Chicken skin with adhering fat
3. Chicken liver
4. Chicken eggs

#### Cattle Substrates:

1. Cow muscle
2. Cow fat
3. Cow liver
4. Cow kidney
5. Cow milk

The following substrates representing different sample types will be analyzed for this method validation study:

Chicken Eggs

Cow Kidney

Cow Fat

Egg data will suffice for milk. Cow kidney data will suffice for liver (chicken and cow) and muscle (chicken and cow). Cow fat will suffice for chicken skin with adhering fat.

Chicken eggs will be obtained from the untreated control (UTC) samples from the Fipronil Chicken Feeding Study (Rhône-Poulenc Ag Company Study Number US93V04R). Cow kidney and cow fat will be obtained from the UTC samples from the Fipronil Cow Feeding Study (Rhône-Poulenc Ag Company Study Number US93V03R). The UTC samples will be supplied by Rhône-Poulenc Ag Company. Source, identification, storage location, and conditions of the UTC samples will be included in the study file.

Rhône-Poulenc crop room personnel will homogeneously grind and handle the UTC substrates according to appropriate Rhône-Poulenc Ag Company SOP guidelines. Analytical samples will be labeled with sample identification, substrate type, study number, and date of collection.

### 1.2 STORAGE

The UTC samples will be stored frozen below 0 °C except during analysis.

## PART 2 - STUDY DESIGN

### 2.1 EXPERIMENTAL DESIGN

The following criteria will be investigated in this method validation.

- Method detection limit (MDL) and limit of quantitation (LOQ)
- Precision and accuracy of the method
- Confirmatory method
- Extraction efficiency
- Ruggedness
- Specificity
- Time requirements
- Range of linearity

Control of Bias: All analytical subsamples will be taken from a single homogeneously ground composite untreated sample. Replicate analysis will be performed.

### 2.2 EXPERIMENTAL METHOD

The samples will be analyzed according to the analytical procedure described in Part 3, Analytical Information.

### 2.3 EXPERIMENTAL PROCEDURE

UTC bulk samples that are known to be free from fipronil residues will be used from the Fipronil animal feeding studies.

EACH SUBSTRATE WILL BE ANALYZED IN THE FOLLOWING STEPS:

#### 2.3.1 Method Detection Limit and Limit of Quantitation

1. Analyze two UTC samples and four UTC samples fortified with the standard mixture of fipronil and its metabolites at a level of estimated MDL.

Note: The level of estimated MDL depends on the signal to noise ratio or apparent residues of fipronil and its metabolites detected in the UTC samples. Previous method development indicated a 5 ppb will be appropriate for the estimated MDL. Thus, the fortified sample will be fortified with 1.0 mL of 0.125 µg/mL of the standard mixture of fipronil and its metabolites into a 25.0 gram

of UTC sample. This will give the estimated MDL of 5 ppb (ng/g).

2. Analyze, on a different day, three UTC samples and three UTC samples fortified at the same standard spike level in step 1.
3. Determine the average apparent residue of each analyte in the five UTC samples from steps 1 and 2 (Section 2.3.1), and sample standard deviation (S.D.) of each analyte in the seven spiked samples from steps 1 and 2 (Section 2.3.1).
4. Determine MDL of each analyte by the summation of the average residue of the UTC samples and three times the sample standard deviation of the spiked samples.

The determined 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.

5. Determine LOQ of each analyte by the summation of the average residue of the UTC samples and ten times the sample standard deviation of the spiked samples.

The determined LOQ is defined as the level of analyte in the substrates above which quantitative results are obtained with a specified degree of confidence.

#### 2.3.2 Accuracy and Precision of the Method

1. Analyze one UTC sample, three UTC samples fortified at a level near the LOQ, and two UTC samples fortified at a level near 5 times the LOQ.
2. Analyze, on a different day, one UTC sample, two UTC samples fortified at a level near the LOQ, and three UTC samples fortified at a level near 5 times the LOQ.
3. Determine the percent recoveries and the mean of percent recoveries, at each spike level for each analyte, in steps 1 and 2 of Section 2.3.2. The mean will describe the accuracy of the analytical method. The target for mean recoveries is between 70% to 120%. Recoveries outside of the target range will also

be reported, as indicative of method accuracy at the respective spike level.

4. Determine the relative standard deviation (RSD) of the percent recoveries at each spike level for each analyte in steps 1 and 2 of Section 2.3.2. These values describe the precision of the analytical method. The target RSD is less than 20%. RSDs greater than 20% will also be reported, as indicative of method precision at the respective spike level.

### **2.3.3 Confirmatory Method**

Qualitative confirmation of residues will be investigated by processing three samples through the method, but substituting one or more of the following steps for the step(s) specified in the method:

1. Columns with different stationary phase
2. Selective detector
3. GC versus HPLC

Some of the extracts from the accuracy and precision testing in section 2.3.2, or extraction efficiency testing Section 2.3.4, may be used for this purpose.

Note: Analysis of one substrate should represent the confirmatory testing for all animal substrates.

### **2.3.4 Extraction Efficiency**

Extraction efficiency will be investigated by processing triplicate animal samples containing grown-in residues of radiolabelled fipronil and one untreated animal sample through the method of analysis. Tissues containing grown-in residues of radiolabelled fipronil will be obtained from the Fipronil chicken metabolism study (Study Number P91/296) or from the two Fipronil goat metabolism studies (Study Numbers P91/125 and P92/125). The extracts will be counted to determine extracted activity. The remaining tissue will be oxidatively combusted to determine unextracted activity. The percent of extracted radioactivity will be determined by dividing the extracted activity by the total activity (sum of extracted and unextracted). This will be compared

to the percent of extracted radioactivity in the metabolism study. If the percent of extracted activity using the method of analysis is at least 90% of the extracted activity in the metabolism study, then the extraction efficiency is acceptable.

If residues are sufficiently high to permit quantitation by the method of analysis, the three extracts will be processed through the entire method. Acceptable extraction efficiency is demonstrated if the average residues for the three test substances in the three replicate samples determined by the method of analysis are within 20% of the residues determined in the metabolism study. If the residues in the tissues from the metabolism study are not sufficiently high for accurate measurement by the method of analysis, the percent of extracted radioactivity, determined above will be compared with the percent of extracted activity of toxicologically significant residues.

Note: Analysis of one substrate will represent the extraction efficiency for all animal substrates.

#### **2.3.5 Ruggedness**

Ruggedness will be qualitatively evaluated and reported by noting any critical steps in the method and any known interchangeability of equipment, reagents, solvents, or techniques. Any known critical steps or equipment, discovered during method development and validation, will be investigated by varying the experimental conditions. The results of these investigations will be reported.

#### **2.3.6 Specificity**

Method specificity will be assessed by:

1. Representative available registered pesticides, which may be present in animal substrates, will be chromatographed under the GC conditions specified in the method.
2. Any pesticide with a retention time within 0.10 minutes of the retention times of the analytes will be processed through the entire method of analysis. Any pesticide recovered through the method, with a retention time within 0.10 minutes of the retention times of the analytes will be reported as a possible interference.

Note: Analysis of one substrate should represent the specificity testing for all substrates because the analytical procedure for all substrates is essentially identical.

**2.3.7 Time Requirements:**

The time period for completion of a set of samples will be determined.

**2.3.8 Range of Linearity:**

Range of linearity will be investigated by determining recoveries at various fortification levels. At least five spiked samples and one recovery must be analyzed at the limit of linearity. For acceptable linearity, the average recovery at the highest fortification level will be within one standard deviation of the average recovery at 5 X LOQ.

**PART 3 - ANALYTICAL INFORMATION**

**3.1 ANALYTICAL STANDARD:** The following compounds will be used for standard calibration and fortification:

**MB46030 (fipronil):** 5-amino-1-(2,6-dichloro-4-trifluoromethylphenyl)-3-cyano-4-trifluoromethanesulphonylpyrazole

Purity: 99.4%  
Log Number: 030051  
Reference No.: AJK 232/C2  
CAS No.: 120068-37-3  
Expiration Date: October 1995

**MB45950:** 5-amino-1-(2,6-dichloro-4-trifluoromethylphenyl)-3-cyano-4-trifluoromethyl-thio-pyrazole

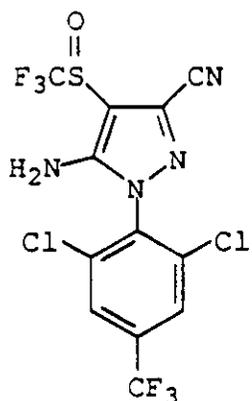
Purity: 99.2%  
Log Number: 029853  
Reference No.: JJW 2120  
CAS No.: 120067-83-6  
Expiration Date: May 1996

MB46136:

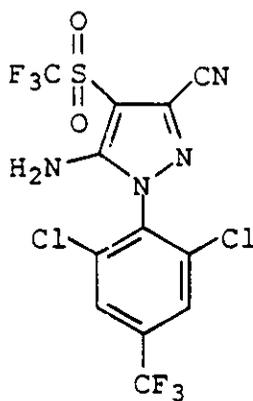
5-amino-1-(2,6-dichloro-4-trifluoromethylphenyl)-3-cyano-4-trifluoromethyl-sulphonyl-pyrazole

Purity: 100.0%  
Log Number: 029493  
Reference No.: 49EAR79  
CAS No.: 120068-36-2  
Expiration Date: April 1995

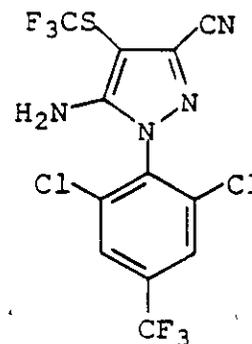
### 3.2 STRUCTURES OF TEST SUBSTANCES



MB 46030



MB 46136



MB 45950

### 3.3 SOLUBILITY CONSIDERATION

No GLP solubility data on the standard solution of fipronil and its metabolites in acetonitrile has been established. However, the recoveries of the standards in this method validation study over a range of fortification levels will demonstrate the solubility of the standards in the acetonitrile.

### 3.4 STABILITY CONSIDERATION

The stability of fipronil and its metabolites in standard solutions will be determined. The standard solutions will be prepared and stored below 0 °C. When new standard solutions are prepared, the new standard solutions will be used to determine the concentrations of the previous standard solutions.

This standard solution stability data will be included in the study notebook or study file.

### 3.5 COMPOUND TO BE DETERMINED

Fipronil and its metabolites listed in Section 3.1.

### 3.6 METHOD OF ANALYSIS

The method of analysis for the determination of fipronil and its metabolites in animal substrates will be based on the method developed by T. W. Robinson, October 18, 1993, and attached in Appendix A. The details of method are listed below..

### 3.7 METHOD SUMMARY

Residues are extracted from animal substrates with 30% acetone in acetonitrile. Following a charcoal/silica gel/florisil column cleanup, residues are quantified by gas chromatography using a Ni<sup>63</sup> electron capture detector.

### 3.8 EQUIPMENT/REAGENTS:

- Aluminum Crimp-top Seal with Teflon/silicone/teflon septum, Cat. No. 200-148, Sun Brokers, Inc., or equivalent
- Bottles, Nalgene 250 mL wide-mouth with screw cap (Nalge Cat. No. 2189-0008), or equivalent
- Buchner Funnel, 85 mm diameter, Coors Cat. 60242, or equivalent
- Chromatographic Column, about 25 cm long, about 1.4 cm ID and about 250 mL round ball reservoir
- Filter Paper, Whatmann Filter No. 1, 70 mm diameter, WWR Cat. No. 28450-069, or equivalent
- Gas Chromatograph, Hewlett Packard 5890 Series II or equivalent, equipped with <sup>63</sup>Ni electron capture detector model G1223A or equivalent
- Graduated cylinder, 250 mL with ground glass joint § 27 stopper, Fisher, or equivalent

- Homogenizer, Waring Model 36BL12, or equivalent
- Shaker, Atlab (Arthur H. Thomas Scientific Co.), or equivalent.
- Water Bath, Precision Model 186 (GCA Corp) or equivalent.
- Acetone, B&J ChromPure™, Burdick & Jackson, CAS 67-64-1, Cat. No. CP80100-4.
- Acetonitrile UV, High Purity Solvent, Burdick & Jackson, CAS 75-05-8, Cat. No. 015-4, or equivalent
- 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
- Celite ® 503 diatomaceous earth, J. T. Baker Co. Cat. No. E406-08, or equivalent.
- Florisil ®, 60-100 mesh, (Mallinkrodt Ca . No. , or equivalent.
- Methanol, B&J ChromPure™, Burdick & Jackson, CAS 67-56-1, Cat. No. 80150-4, or equivalent.
- Silica Gel, Woelm, 63-200 microns, or equivalent
- Extraction Solution: Add 300 mL HPLC grade acetone to 700 mL UV grade acetonitrile in a 1000 mL Erlenmeyer flask.

### 3.9 PROCEDURES

#### 3.9.1 Standard Solution Preparation of Each Standard

Using an analytical balance, weigh accurately ( $\pm 0.1$  mg) MB46030, MB45950, and MB46136 analytical standards individually into separate 100 mL volumetric flasks to obtain target weight of 100.0 mg. Dissolve in UV grade acetonitrile, dilute and mix well before bringing to the 100 mL mark. Mix well. These solution contains 1000  $\mu\text{g}/\text{mL}$  of the standards in acetonitrile.

### 3.9.2 Standard Mixture Preparation

Pipette 1.00 mL from each of the three 1000 µg/mL solutions above into a 100 mL volumetric flask. Dilute to volume with UV grade acetonitrile. This is a 10 µg/mL mixed standard containing 10 µg/mL each of MB46030, MB45950, and MB46136 in acetonitrile.

By further dilution of the 10 µg/mL mixed standard, prepare a series of mixed standards to serve as both spiking and calibration solutions.

### 3.9.3 Sample/Extract Preparation

#### 1. SAMPLE PREPARATION

1.1 Cut muscle, skin, and fat samples into small pieces. Place chopped sample and crushed dry ice into a blender jar and blend into a finely ground homogeneous mixture. Dry ice must sublime before subsample can be removed.

1.2 Cut liver and kidney samples into small pieces and allow to reach room temperature. Place chopped sample into a blender jar and blend until a homogeneous slurry is achieved.

1.3 Allow eggs to reach room temperature. Remove egg from shell and beat until a homogeneous mixture between yolk and white is achieved.

#### 2. EXTRACTION

2.1 Weigh 25 g of control sample into a 250 mL Nalgene screw-capped bottle. Fortification for the purpose of recovery determination should be done at this point. Spiked samples should set at least 10 minutes before the addition of the extraction solution.

2.2 Add 100 mL of extraction solution (30% acetone in acetonitrile) and approximately 20 g sodium sulfate (5 g Celite is also added to beef fat and chicken skin). Shake vigorously by hand for about 15 seconds then place on mechanical shaker for at least 15 minutes.

2.3 Filter through Whatman #1 filter paper into a side arm flask using suction and rinse with 30-40 mL acetonitrile. Pour the filtrate into a 250 mL graduated cylinder and bring up to 150 mL volume with acetonitrile. Return this to the 250 mL Nalgene bottle.

### 3. COLUMN CHROMATOGRAPHY CLEANUP

- 3.1 Plug a 15 mm i.d. glass chromatography column with glass wool. Add approximately 2.0 g 60-80 mesh activated florisil (florisil is activated by heating overnight at about 130 degrees C), 2.0 g 63-200  $\mu\text{m}$  silica gel, 2.0 g 20-40 mesh activated carbon, and 6.0 g sodium sulfate. Tap the column after the addition of each to settle the packing.
- 3.2 Wash above column with 25-30 mL each methanol, acetone, and UV grade acetonitrile in that order. Discard the washes.
- 3.3 Place a 250 mL Erlenmeyer flask under the column. Pipet a 6.0 mL aliquot of the sample extract onto the column. When this reaches the top of the sodium sulfate, add approximately 75 mL acetonitrile. (If column stops dripping due to air pockets in the charcoal and sodium sulfate, tap on the side until air bubbles are released.)
- 3.4 Place the Erlenmeyer flask in a warm water bath (approximately 35-40 degrees C) and evaporate to just dryness using a stream of nitrogen.
- 3.5 Dilute to desired volume with UV grade acetonitrile.

#### 3.9.4 Analysis

1. Hewlett Packard 5890 Gas Chromatograph, or equivalent.
2. Ni<sup>63</sup> Electron Capture Detector
3. Hewlett Packard 3396 Integrator, or equivalent
4. Column: J&W Scientific DB-1701, 15m X 0.32 mm i.d., 0.25  $\mu\text{m}$  film thickness
5. Oven Temperature: Initial temp 50 degrees C. Hold for 1 minute. Ramp 70 degrees C per minute to 200 degrees. Hold for 22 minutes. Ramp 70 degrees C per minute to 230 degrees C. Hold for 17 minutes.
6. Injection Temperature: 280 degrees C.
7. Detector Temperature: 300 degrees C.

8. Helium Carrier Gas: 2.0 mL/min.
9. Makeup Gas: 50 to 60 mL/min 5% Argon in Methane.
10. Approximate Retention Times:

MB46950	18 minutes
MB46030	19.5 minutes
MB46136	33.5 minutes

11. Injection Volume: 1.0 µL.
12. Inlet Liner: 4 mm i.d., nominal volume 900 µL, borosilicate glass with silanized glass wool plug (HP Part # 5062-3587).
13. Splitless injection with split vent off for 30 seconds.
14. Note: Detector parameters and recorder attenuation should be adjusted such that a 1.0 µL injection of an 0.002 µg/mL standard gives a measurable and reproducible peak. Several standards should be injected prior to the actual GC analysis of the set to condition the column and to "bake off" any residual contaminants from previous injections.

#### 3.9.4 Calculations

1. Linear regression is used to generate calibration curves for MB45950, MB46030, and MB46136. At least 4 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 (or heights) obtained are within the area (or height) range between the lowest and highest standards injected.
2. Linear regression coefficients are calculated on ng/mL injected versus peak area (or height). The data from the analytical standards are fit to a linear model:

$$y = a + bx$$

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

The coefficient of determination,  $R^2$ , is also calculated.

3. Calculate the concentration of analyte in the injected solution by:

$$\text{Conc in injected solution} = \frac{(\text{peak height or area}) - (\text{calibration line intercept})}{\text{calibration line slope}}$$

4. Calculate the concentration of analyte in the original sample by:

$$\text{Conc in original sample} = \frac{(\text{conc in injected soln}) \times (\text{dilution volume, mL})}{\text{weight of original sample}}$$

5. Calculate the percent recovery by:

$$\text{Percent Recovery} = \frac{(\text{ppm found in spiked UTC}) - (\text{ppm found in UTC})}{\text{ppm added to UTC}}$$

#### PART 4 - RECORD KEEPING / QUALITY ASSURANCE

##### 4.1 DATA AND REPORTS

1. All raw data and the original signed protocol will be maintained in the study file. This data include the protocol amendments, protocol deviations, laboratory notebooks, analytical standard solution preparation, sample chain custody sheets, sample work sheets, chromatograms, calibration curves, and any other appropriate data generated.
2. A final report following the guidelines of United States Environmental Protection Agency PR Notice 86-5, approved and signed by the Study Director and sufficient for submission to EPA will be prepared.

##### 4.2 ARCHIVE STATEMENTS:

1. Study records to be maintained: Records to be maintained for the study include all raw data, observations recorded during the conduct of the

study, documentation, chromatograms, specimens, and study related correspondence. This includes a description of equipment (with serial numbers) used during the conduct of the study. All characterization data and any shipping records shall be retained.

2. Document archives: Upon completion of the study, the study records, protocol and amendments, and the final report and amendments shall be retained in the Rhône-Poulenc Ag Company document archives. If it is necessary to substitute a copy for an original record, it will be certified as an exact copy.
3. Chemical archives: Upon completion of the study, samples of the applicable test, reference, and control substances shall be retained in the Rhône-Poulenc Ag Company chemical archives for as long as the quality of the materials afford evaluation or until product registration has ended, whichever occurs first.
4. UTC samples: Upon completion of the study, unused sample substrates will be maintained in freezers until QA verification, at which time the Study Director will determine the discard date.
5. Protocol amendments: Planned changes to the approved protocol shall be documented by amendments that clearly describe the change, justification for the change, and impact on the study. Amendments will be signed and dated by the Study Director and Group Leader. Copies of amendments will be sent to the Quality Assurance Unit.
6. Protocol deviations: Protocol deviations, which are one time and unplanned deviations from the protocol shall be documented in the study records, noting the nature of the deviation, potential effect or impact on the study, and corrective action if required. Protocol deviations are signed or initialed by the Study Director, but because they are unplanned events, are usually accepted after the fact.
7. SOP and GLP deviations: All deviations from Standard Operating Procedures or Good Laboratory Practices must be authorized or accepted by the Study Director and documented in the study records, noting the nature of the deviation, potential effect on the study, and corrective action.

#### 4.3 STATISTICAL ANALYSIS

Statistical analysis of the sample data will be limited to determination of mean, standard deviation and relative standard deviation.

#### 4.4 PERSONNEL

Study Director: J. R. Hudson

Study Supervisor: T. W. Hunt

#### 4.5 SAFETY

All available appropriate Material Safety Data Sheets will be available to the study personnel during the conduct of the study.

APPENDIX A

METHOD OF ANALYSIS FOR THE DETERMINATION OF FIPRONIL (MB46030), AND IT'S  
METABOLITES (MB45950 AND MB46136) IN MILK, EGGS, LIVER, KIDNEY, MUSCLE AND  
FAT TISSUES

AUTHOR: T. W. ROBINSON

DATE: OCTOBER 18, 1993

**METHOD OF ANALYSIS FOR THE DETERMINATION OF FIPRONIL,  
(MB46030) AND ITS METABOLITES (MB45950 AND MB46136) IN MILK,  
EGGS, LIVER, KIDNEY, MUSCLE AND FAT TISSUES**

**INTRODUCTION**

A method of analysis was developed to determine possible residues of fipronil and its metabolites MB45950 and MB46136 in muscle, fat, liver, kidney (except poultry) milk and eggs. The validity of the method was demonstrated by fortifying samples over the range of 10 to 100 ppb with MB46030, MB45950 and MB46136.

Residues are extracted from the substrates with 30% acetone in acetonitrile. Following a charcoal, silica gel, florisil column cleanup, residues are quantified by gas chromatography using a Ni<sup>63</sup> electron capture detector.

**REAGENTS**

1. Acetone, B&J ChromPure™, HPLC grade (Burdick & Jackson)
2. Acetonitrile, UV grade, (Burdick & Jackson)
3. Activated carbon, Darco®, granular 20-40 mesh (Aldrich Chemical Company)
4. Celite, (J. T. Baker, Inc.)
5. Florisil, 60-100 mesh (Mallinckrodt)
6. Methanol, B&J ChromPure™, HPLC grade, (Burdick & Jackson)
7. Silica Gel Woelm®, 63-200 µm, (Woelm Pharma)
8. Sodium sulfate, granular, (Mallinckrodt)
9. MB45950, (Rhône-Poulenc)
10. MB46030, (Rhône-Poulenc)
11. MB46136, (Rhône-Poulenc)

**EQUIPMENT**

1. Model 186 Precision Water Bath (GCA Corp.) or equivalent.
2. Atlab Shaker, (Arthur H. Thomas Co.) or equivalent
3. Waring blender or equivalent
4. Nalgene® 250 mL screw-capped bottles (Nalge Co. # 2199-0008)

**EXTRACTION SOLUTION**

1. Add 300 mL HPLC grade acetone to 700 mL UV grade acetonitrile in a 1000 mL Erlenmeyer flask.

**STANDARD SOLUTIONS**

1. Weigh 0.1000 g of MB46030, MB45950 and MB46136 individually into 100 mL volumetric flasks and dilute to volume with UV grade acetonitrile. Concentration of these standards is 1000 µg/mL.
2. Withdraw a 1.0 mL aliquot from each of the three 1000 µg/mL standards above and put into a 100 mL volumetric flask. Dilute to volume with UV grade acetonitrile. This is a 10 µg/mL mixed standard.
3. By further dilution of the 10 µg/mL mixed standard, prepare a series of mixed standards to serve as both spiking and calibration solutions.

4. After preparation, standards should be transferred from the volumetric flasks into screw capped brown glass bottles to prevent possible photodegradation.
5. Store standards in refrigerator or freezer when not in use.

#### PROCEDURE

##### Sample Preparation

Cut muscle, skin and fat samples into small pieces. Place chopped sample and crushed dry ice into a blender jar and blend into a finely ground homogeneous mixture. Dry ice must sublime before subsample can be removed.

Cut liver and kidney samples into small pieces and allow to reach room temperature. Place chopped sample into a blender jar and blend until a homogeneous slurry is achieved.

Allow eggs to reach room temperature. Remove egg from shell and beat until a homogenous mixture between yolk and white is achieved.

##### Extraction and Cleanup

1. Weigh 25 g of control sample into a 250 mL Nalgene screw-capped bottle. Fortification for the purpose of recovery determination should be done at this point. Spiked samples should set at least 10 minutes before the addition of the solvent solution.
2. Add 100 mL extraction solution (30% acetone in acetonitrile) and approximately 20 g sodium sulfate (5 g celite is also added to beef fat and chicken skin.) Shake vigorously by hand for about 15 seconds then place on mechanical shaker for at least 15 minutes.
3. Filter through Whatman #1 filter paper into a side arm flask using suction and rinse with 30-40 mL acetonitrile. Pour filtrate into a 250 mL graduated cylinder and bring up to 150 mL volume with acetonitrile. Return this to the 250 mL Nalgene bottle.
4. Plug a 15mm i.d. glass chromatography column with glass wool. Add approximately 2.0 g 60-80 mesh activated florisil<sup>1</sup>, 2.0 g 63-200 µm silica gel, 2.0 g 20-40 mesh activated carbon and 6.0 g sodium sulfate. Tap the column after the addition of each to settle the packing.
5. Wash above column with 25-30 mL each methanol, acetone, and UV grade acetonitrile in that order. Discard the washes.
6. Place a 250 mL Erlenmeyer under the column. Pipet a 6.0 mL aliquot of the sample extract onto the column. When this reaches the top of the sodium sulfate, add approximately 75 mL acetonitrile<sup>2</sup>.
7. Place the Erlenmeyer in a warm water bath (approximately 35-40°C) and evaporate to just dryness.
8. Dilute to desired volume with UV grade acetonitrile.

<sup>1</sup> Florisil is activated by heating overnight at about 130°C.

<sup>2</sup> If column stops dripping due to air pockets in charcoal and sodium sulfate, tap on sides until air bubbles are released.

Instrumentation

1. Hewlett Packard 5890 Gas Chromatograph, or equivalent
2. Ni<sup>63</sup> Electron Capture Detector
3. Hewlett Packard 3396 Integrator, or equivalent
4. Column: J&W Scientific DB-1701, 15m x .32mm i.d.  
0.25 µm film thickness
5. Oven Temperature: Initial temp 50°C - hold for 1 minute  
Ramp 70°C/min to 200°C - hold for 22 minutes  
Ramp 70°C/min to 230°C - hold for 17 minutes
6. Injection Temperature: 280°C
7. Detector Temperature: 300°C
8. Helium (carrier): 2.0 mL/min.
9. 95% Methane/Argon: 50 to 60 mL/min.  
(make-up)
10. Approx. Ret. Times: MB 46950 18 minutes  
MB 46030 19.5 minutes  
MB 46136 33.5 minutes
11. Injection Volume: 1.0 µL
12. Inlet Liner: 4-mm i.d. nominal volume 900 µL, borosilicate  
glass with silanized glass wool plug  
(HP Part # 5062-3587)
13. Splitless injection with split vent off for 30 seconds.

NOTE: Detector parameters and recorder attenuation should be adjusted such that a 1.0 µL injection of an 0.002 µg/mL standard gives a measurable and reproducible peak.

Several standards should be injected prior to the actual GC analysis of the set to condition the column and to "bake off" any residual contaminants from previous injections.



RESIDUE STUDY PROTOCOL AMENDMENT

I. AMENDMENT NUMBER: One (1)

II. STUDY IDENTIFICATION:

Study Number: EC-94-258

Study Title: Fipronil - Validation of Method of Analysis for Fipronil and its Metabolites in Animal Tissues

Study Director: J. R. Hudson

III. LOCATION OF CHANGE:

Page 3 of Protocol: "TEST SYSTEM IDENTIFICATION"

IV: DESCRIPTION OF CHANGE

Untreated control samples of chicken eggs, cow kidney, and cow fat will not be obtained from the UTC samples from the Fipronil Chicken and Cow feeding studies (study numbers US93V04R and US93V03R, respectively). These samples will be obtained from commercial sources. The sources will be identified in the study records.

V. REASON FOR CHANGE AND EFFECT ON STUDY:

Insufficient amount of samples remain from the feeding studies for use in this study. Using other sources for these substrates will have no adverse effect on the study.

VI. DISTRIBUTION:

J. R. Hudson, Study Director  
A. A. Gemma, Quality Assurance Unit

VII: APPROVAL:

J R Hudson  
J. R. Hudson  
Study Director

April 28 1994  
Date

Timothy W Hunt  
T. W. Hunt  
Group Leader

28 April 94  
Date

**RESIDUE STUDY PROTOCOL AMENDMENT**

I. **AMENDMENT NUMBER:** Two (2)

II. **STUDY IDENTIFICATION:**

Study Number: EC-94-258

Study Title: Fipronil - Validation of Method of Analysis for Fipronil and its Metabolites in Animal Tissues

Study Director: J. R. Hudson

III. **LOCATION OF CHANGE:**

Page 5 of Protocol, section 2.3.2: "Accuracy and Precision of the Method".

IV: **DESCRIPTION OF CHANGE**

The protocol, section 2.3.2 states that accuracy and precision will be measured by analyzing samples fortified near the LOQ and near 5 times the LOQ. The protocol will be followed with the exception of the spike levels. The following spike levels will be substituted for "levels near the LOQ" and "levels near 5 times the LOQ":

SUBSTRATE	Substitute Spike Level for "near the LOQ"	Substitute Spike Level for "near 5 times the LOQ"
Chicken Eggs	10 ppb (each analyte)	25 ppb (each analyte)
Cow Kidneys	10 ppb (each analyte)	25 ppb (each analyte)
Cow Fat	20 ppb (each analyte)	100 ppb (each analyte)

V. **REASON FOR CHANGE AND EFFECT ON STUDY:**

The calculated LOQs for each analyte in eggs and kidneys are near 5 ppb, which was the fortification level used in the determination of LOQ. It is unnecessary to run more recoveries at that level. The spike levels for determination of accuracy and precision in cow fat are near the LOQ and near 5 times the LOQ, as stated in the protocol. The change will have no adverse effect on the study. The addition of the 10 ppb spike levels for eggs and kidneys will provide additional information on the performance of the method near the LOQ.

VI. **DISTRIBUTION:**

J. R. Hudson, Study Director  
A. A. Gemma, Quality Assurance Unit

VII: APPROVAL:

J R Hudson  
J. R. Hudson  
Study Director

June 14, 1994  
Date

Timothy W Hunt  
T. W. Hunt  
Group Leader

20 June 94  
Date

**RESIDUE STUDY PROTOCOL AMENDMENT**

I. **AMENDMENT NUMBER:** Three (3)

II. **STUDY IDENTIFICATION:**

Study Number: EC-94-258

Study Title: Fipronil - Validation of Method of Analysis for Fipronil and its Metabolites in Animal Tissues

Study Director: J. R. Hudson

III. **LOCATION OF CHANGE:**

Page 9 of Protocol, section 3.4: "Stability Consideration".

IV: **DESCRIPTION OF CHANGE**

The protocol states that standard solutions will be stored below 0 °C. The standards will be stored in a refrigerator, below 8 °C.

V. **REASON FOR CHANGE AND EFFECT ON STUDY:**

Based on information obtained in the certificates of analysis for the analytes, the compounds are stable at refrigerator temperatures and need not be stored in a freezer. In addition, as stated in section 3.4 the stability of these solutions will be determined by comparing them to freshly prepared solutions. Therefore, this change will have no adverse effect on the study.

VI. **DISTRIBUTION:**

J. R. Hudson, Study Director  
A. A. Gemma, Quality Assurance Unit

VII: **APPROVAL:**

J R Hudson  
J. R. Hudson  
Study Director

June 21, 1994  
Date

Timothy W Hunt  
T. W. Hunt  
Group Leader

27 June 94  
Date

**RESIDUE STUDY PROTOCOL AMENDMENT**

I. **AMENDMENT NUMBER:** Four (4)

II. **STUDY IDENTIFICATION:**

Study Number: EC-94-258

Study Title: Fipronil - Validation of Method of Analysis for Fipronil and its Metabolites in Animal Tissues

Study Director: J. R. Hudson

III. **LOCATION OF CHANGE:**

Page 6 of Protocol, section 2.3.2: "Extraction Efficiency".

IV: **DESCRIPTION OF CHANGE**

This amendment clarifies and expands this section of the protocol. In addition to the samples containing grown-in radiolabelled fipronil residues, one untreated beef fat sample and two untreated beef fat samples fortified at the approximate residue level of the samples containing grown-in radiolabelled fipronil residues will be processed through the method. Subsamples of the tissue containing grown-in radiolabelled fipronil residues will be oxidatively combusted to determine the starting total activity in the tissue. In addition, aliquots of the final solution (from the first dilution in step 8 in the method, will be counted for radioactivity. Subsequent dilutions will not be counted.

V. **REASON FOR CHANGE AND EFFECT ON STUDY:**

This amendment clarifies and expands this section of the protocol. These details will improve the quality of the results and will have no adverse impact on the study.

VI. **DISTRIBUTION:**

J. R. Hudson, Study Director  
A. A. Gemma, Quality Assurance Unit

VII: **APPROVAL:**

J. R. Hudson  
J. R. Hudson  
Study Director

June 29, 1994  
Date

T. W. Hunt  
T. W. Hunt  
Group Leader

29 June 94  
Date

**RESIDUE STUDY PROTOCOL AMENDMENT**

I. **AMENDMENT NUMBER:** FIVE (5)

II. **STUDY IDENTIFICATION:**

Study Number: EC-94-258

Study Title: Fipronil - Validation of Method of Analysis for Fipronil and its Metabolites in Animal Tissues

Study Director: J. R. Hudson

III. **LOCATION OF CHANGE:**

Pages 6 and 7 of Protocol, section 2.3.4: "Extraction Efficiency".

IV: **DESCRIPTION OF CHANGE**

This amendment changes the calculations for extraction efficiency. The extraction efficiency will be determined by dividing the amount of activity in the extract by the amount of activity in the starting (unextracted) sample. The percent radioactivity recovery in the final solution will also be reported. This is obtained by dividing the amount of activity in the final solution by the theoretical amount of activity in the final solution.

V. **REASON FOR CHANGE AND EFFECT ON STUDY:**

Both of these changes were made to improve the reporting of the extraction performance of the method of analysis.

Calculation of percent extracted radioactivity by division of extracted activity by activity in the starting (unextracted) sample is a more exact and less complicated process than that proposed in the original protocol. The amount of activity in the unextracted sample (following extraction) is very small and somewhat imprecise. The imprecision is due to the low level of activity remaining in the sample and the difficulty of obtaining a homogeneous subsample for measurement from the filter cake which contains sample, sodium sulfate, and celite.

Calculation of percent activity in the final solution will give another measure of the overall efficiency of the method.

Both of these changes will have no adverse effect on the study.

VI. **DISTRIBUTION:**

J. R. Hudson, Study Director  
A. A. Gemma, Quality Assurance Unit

VII: APPROVAL:

J R Hudson  
J. R. Hudson  
Study Director

August 4, 1994  
Date

Timothy W Hunt  
T. W. Hunt  
Group Leader

5 August 94  
Date

**RESIDUE STUDY PROTOCOL AMENDMENT**

I. **AMENDMENT NUMBER:** Six (6)

II. **STUDY IDENTIFICATION:**

Study Number: EC-94-258

Study Title: Fipronil - Validation of Method of Analysis for Fipronil and its Metabolites in Animal Tissues

Study Director: J. R. Hudson

III. **LOCATION OF CHANGE:**

Page 8 of Protocol, section 2.3.8: "Linearity".

IV: **DESCRIPTION OF CHANGE**

The acceptance criterion for average recovery at the highest fortification level is changed from within one standard deviation to within two standard deviations of the average recovery at 5 X LOQ.

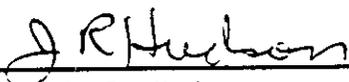
V. **REASON FOR CHANGE AND EFFECT ON STUDY:**

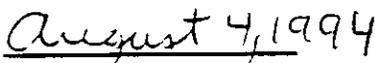
The acceptance criterion stated in the original protocol is too stringent. Acceptance of average recoveries within one standard deviation of the average recovery at 5 X LOQ would statistically exclude 33% of the expected average recoveries coming from the same population. The change to 2 standard deviations would include 95% of the average recoveries expected due to normal statistical variability. This change has no adverse effect on the study.

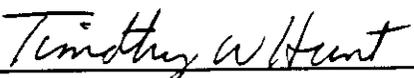
VI. **DISTRIBUTION:**

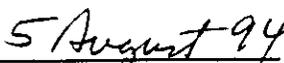
J. R. Hudson, Study Director  
A. A. Gemma, Quality Assurance Unit

VII: **APPROVAL:**

  
\_\_\_\_\_  
J. R. Hudson  
Study Director

  
\_\_\_\_\_  
Date

  
\_\_\_\_\_  
T. W. Hunt  
Group Leader

  
\_\_\_\_\_  
Date

**RESIDUE STUDY PROTOCOL AMENDMENT**

- I. **AMENDMENT NUMBER:** ~~Six~~ (7) *Seven GRH 9-2-94 wrong number.*
- II. **STUDY IDENTIFICATION:**  
Study Number: EC-94-258  
Study Title: Fipronil - Validation of Method of Analysis for Fipronil and its Metabolites in Animal Tissues  
Study Director: J. R. Hudson
- III. **LOCATION OF CHANGE:**  
Protocol amendment Four (4), item III LOCATION OF CHANGE.
- IV. **DESCRIPTION OF CHANGE**  
Amendment 4 incorrectly stated that the location of the change for amendment 4 was in page 6 of the protocol, section 2.3.2: "Extraction Efficiency". The correct location of the change is page 6 of the protocol, section 2.3.4: "Extraction Efficiency".
- V. **REASON FOR CHANGE AND EFFECT ON STUDY:**  
The location of the change for protocol amendment 4 was incorrect. This amendment corrects the location, by identifying the correct section (section 2.3.4) of the protocol. This change has no adverse effect on the study.
- VI. **DISTRIBUTION:**  
J. R. Hudson, Study Director  
A. A. Gemma, Quality Assurance Unit
- VII: **APPROVAL:**

J. R. Hudson  
J. R. Hudson  
Study Director

September 1, 1994  
Date

A. A. Chene  
A. A. Chene  
Group Leader

September 1, 1994  
Date