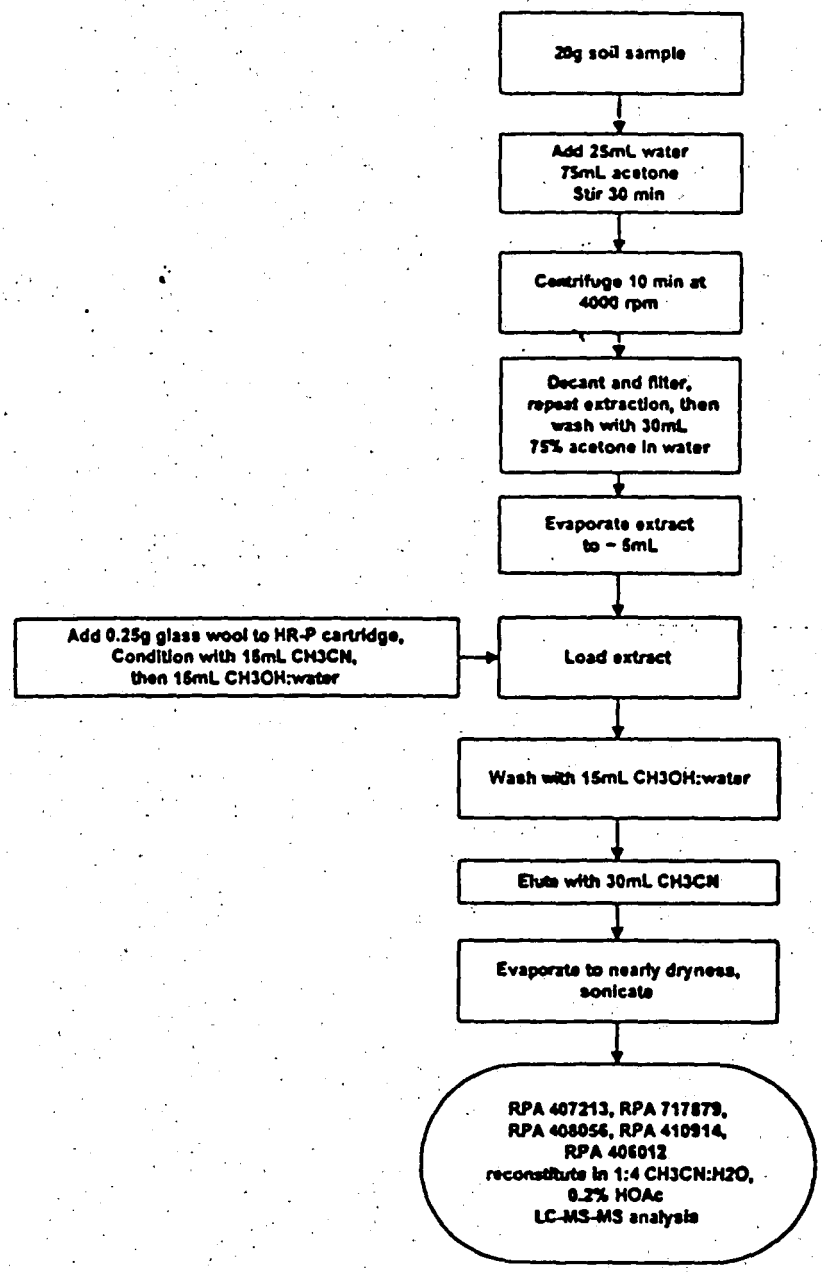
Summary Flowchart of Analytical Method



Fenamidone (RPA 407213) : Method of Analysis for RPA 407213 and Its Metabolites in Soil

I. INTRODUCTION

A. Scope

This method sets forth the procedure for determining the residues of RPA 407213 and its metabolites RPA 717879, RPA 408056, RPA 410914 and RPA 406012 in soil.

B. Principle

An analytical method is described for the determination of residues of RPA 407213 and its metabolites RPA 717879, RPA 408056, RPA 410914 and RPA 406012 in soil. Residues of RPA 407213, RPA 717879, RPA 408056, RPA 410914 and RPA 406012 are extracted from soil by stirring with acetone:water (3:1), and the extract centrifuged, decanted, and filtered. The extract is purified using a polystyrene-divinylbenzene polymer cartridge.

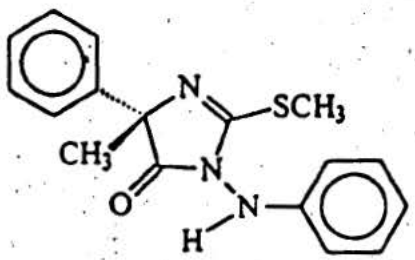
A reversed-phase C18 high performance liquid chromatography (HPLC) column is used to separate the compounds which are then quantified using the multiple reaction monitoring (MRM) mode. A turbo-ion interface is used to introduce the HPLC eluant into the mass spectrometer for analyte analysis. Quantitation is performed by daughter ion detection using liquid chromatography/mass spectrometry (LC/MS/MS) analysis. Quantification of results is based on a comparison of peak areas with those of known standards. The method has been verified at 10, 50 and 500 parts per billion (ppb) for RPA 407213, RPA 717879, RPA 408056, RPA 410914 and RPA 406012 by preparing and analyzing control and fortified samples of soil from Florida, California, North Dakota, and Washington.

C. Method Limits

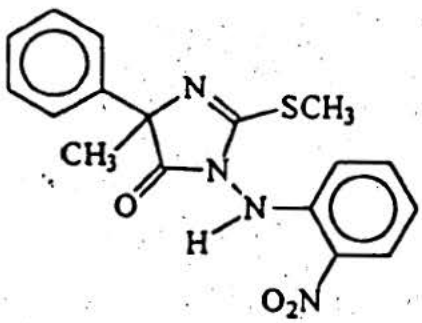
The minimum limits of detection (LOD) and limits of quantification (LOQ) for RPA 407213, RPA 717879, RPA 408056, RPA 410914 and RPA 406012 in each soil type have not been determined. This information will be obtained from the subsequent validation study. The target level for LOQ is 10 ppb for RPA 407213, RPA 717879, RPA 408056, RPA 410914 and RPA 406012.

D. **Chemical Structures**

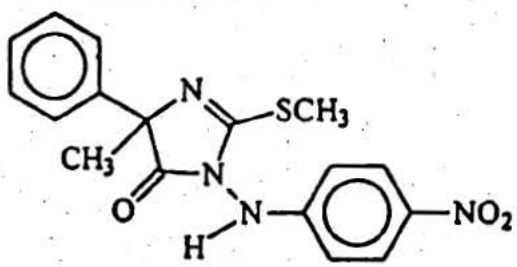
RPA 407213



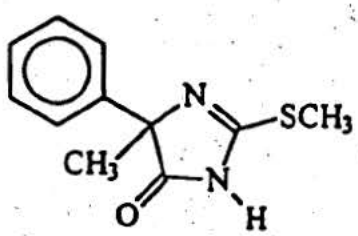
RPA 410914



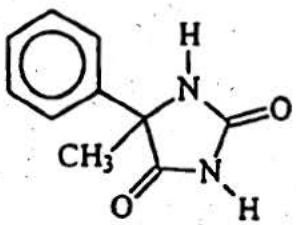
RPA 406012



RPA 408056



RPA 717879



II. MATERIALS

Unless otherwise noted, equivalent brands and/or suppliers can be used.

A. Reagents/Solvents

Acetic Acid Glacial (EM Science, Cat. No. AX0073-14)

Acetone Omni-Solv (EM Science, Cat. No. AX0116-1)

Acetonitrile Omni-Solv (EM Science, Cat. No. AX0142-1)

Methanol Omni-Solv (EM Science, Cat. No. MX0488-1)

Water (HPLC grade)

B. Equipment and Supplies

Adaptor, Bond Elut (Varian, Cat. No. 1213-1001)

Balance :
accuracy ± 0.1 mg (analytical standards) (Mettler AE 200 or equiv)
accuracy ± 0.1 g (samples and chemicals)(Mettler PC 4000 or equiv)

Bottles, amber, 4 oz. (Qorpak, No. 7919)

Bottles, wide mouth, polypropylene, 250mL (Nalge, Part No. 2105-0008)

Cartridges , Chromabond™ HR-P polystyrene-divinylbenzene (0.5 g)
(Machery Nagel, Cat. No. 730111, *no substitute, order from Bodman*)

Centrifuge (Marathon 10K)

Column, HPLC, Alltima C-18, 100mm x 4.6mm id., 5µm particle size
(Alltech, Cat. No. C-6000B, *custom order*)

Guard Column, HPLC, Alltima C18, 5 micron
(Alltech, Cat. No. 96361) optional

Centrifuge Tubes, Blue Max, disposable, polypropylene, 50mL
(Falcon, No. 2098)

Disposable pipettes

Filter paper, glass microfibre, grade GF/C, 7.0cm (Whatman, Cat. No. 1822 070)

Filter Adapter, neoprene crucible holder (VWR Cat. No. 24035-065)

Filter paper, grade 541, 70mm (Whatman, Cat. No. 1541 070)

Flasks, evaporation , flat-bottom, 500mL (Kimble, 25055-500)

Flasks, evaporation , flat-bottom, 125mL (Kimble, 25055-125)

Funnels, Buchner, 83mm (Coors, No. 60242)

Glass wool

Graduated cylinders

Hitachi AS2000 autosampler

Magnetic Stirrer (Corning, Model No. PC-410, Cat. No. 6795-410)

Perkin Elmer Sciex API III+ LC/MS/MS system coupled to an Hitachi L6200 HPLC pump via PE TurboIonSpray interface.

Perkin Elmer Sciex API 300 LC/MS/MS system coupled to an Hewlett Packard HP1100 HPLC via PE Turbo IonSpray interface.

Pipette bulb

Reservoir, empty, 70mL (Varian, Cat. No. 1213-1018)

Rotary vacuum evaporator (Buchi R-124)

SPE cartridge adaptors (University Research Glass, Part No. URG-2440-SPECA)

Septa, 8mm T/S, slit (Sun, 500-870)

Solvent jugs, 4 L brown glass

Stir bars, magnetic, 50.8 x 9.5mm (VWR, Cat. No. 58949-130)

Stopcocks, Luer Lock (Varian, 1213-1005)

Stoppers, glass, 24/40

Syringe, disposable, 3mL (Becton-Dickinson, Cat. No. BD309586)

Syringe filter, Nylon Acrodisc® 13mm, 0.45µm (Gelman, No. 4426)

Traps, rotary evaporator

Ultrasonic bath (Bransonic 52)

Vacuum manifold system for cartridge elution

Vacuum Gauges

Vacuum Pump, Duo Seal (Welch, Model No. 1400)

Varian Vac Elut SPS 24 Vacuum Manifold (Varian, Model 1223-4004)

Vials, clear, 1.5mL (Sun, 200-250)

Vial caps (Sun, 200-292)

Volumetric flasks

Volumetric pipettes

Vortex®-Genie Mixer (Scientific Industries, Model No. K-550-G)

C. Solutions

The following is a list of the solutions used in the analyses of soil. Example procedures for the preparation of each solution are also provided. *Note that the reagent water used in the preparations should be HPLC grade.*

1. Solution of 75 % Acetone in Water
   Using a 1 liter graduated cylinder, transfer ~750 mL of acetone and ~250 mL of water to a 4 L brown glass jug. Mix by shaking. Repeat until the desired quantity has been made.

2. Solution of 50% Methanol in Water
   Using a 1 liter graduated cylinder, transfer ~1000 mL of methanol and ~1000 mL of water to a 4 L brown glass jug. Mix by shaking. Repeat until the desired quantity has been made.

3. Solution of 0.2 % Acetic Acid in Water
   Using a 1 liter graduated cylinder, transfer ~998 mL of water to a 1 L HPLC solvent reservoir. Add ~2 mL acetic acid. Mix by shaking.

4. Solution of 20% Acetonitrile in Water, 0.2 % Acetic Acid
   Using a 1 liter graduated cylinder, transfer ~200 mL of acetonitrile and ~800 mL of 0.2 % acetic acid in water to a 4 L brown glass jug. Mix by shaking. Repeat until the desired quantity has been made.

5. Solution of 20% Acetonitrile in Water
   Using a 1 liter graduated cylinder, transfer ~200 mL of acetonitrile and ~800 mL of water to a 4 L brown glass jug. Mix by shaking. Repeat until the desired quantity has been made.

D. Analytical Standards

Common name/alias: fenamidone/ RPA 407213

Chemical name: (+)-(4S)-4-methyl-2-methylthio-4-phenyl-(1H)-1-phenylamino-2-imidazolin-5-one
(CAS No. 161326-34-7)

Solubility [1]:

acetone:	250 (unit : g/L)
acetonitrile:	86
dichloromethane:	330
methanol:	43
toluene:	40
water:	0.0078

Common name/alias: RPA 408056

Chemical name: 4-methyl-2-methylthio-4-phenyl-2-imidazolin-5-one

Common name/alias: RPA 717879

Chemical name: 4-methyl-4-phenylimidazolidin-2,5-dione

Common name/alias: RPA 410914

Chemical name: (4RS)-4-methyl-2-methylthio-(1H)-1-(2-nitrophenylamino)-4-phenyl-2-imidazolin-5-one

Common name/alias: RPA 406012

Chemical name: (4RS)-4-methyl-2-methylthio-(1H)-1-(4-nitrophenylamino)-4-phenyl-2-imidazolin-5-one

III. FORTIFICATION AND CALIBRATION STANDARD SOLUTIONS

A. Preparation

All the standard solutions must be stored in amber glass bottles, at or below 10°C when not in use. Solutions should be allowed to warm to room temperature prior to use. The following is an example of a procedure to follow in preparing standard solutions. Alternate or additional standards of appropriate weight and volume may be prepared as needed. The "~" symbol indicates approximately. RPA 717879 is kept as a solution separate from the other compounds.

1. Weigh ~0.0200g (corrected for purity) each of RPA 407213, RPA 408056, RPA 717879, RPA 410914, and RPA 406012 into separate 100-mL volumetric flasks and dilute to the marks with acetonitrile. Cap and mix by inversion. The concentration of these stock standards is ~200 µg/mL.

2. a. For the preparation of fortification standards of RPA 407213, RPA 408056, RPA 410914, and RPA 406012, transfer 10 mL of each of the ~200 µg/mL standard solutions, via volumetric class "A" pipettes, to one 100 mL volumetric flask. Dilute to mark with acetonitrile. Cap and mix by inversion. The concentration of this mixed standard is ~20 µg/ mL RPA 407213, RPA 408056, RPA 410914, and RPA 406012.

   b. For the preparation of the fortification standard for RPA 717879 transfer 10 mL of the ~200 µg/mL standard solution, via a volumetric class "A" pipette, to a 100 mL volumetric flask. Dilute to mark with acetonitrile. Cap and mix by inversion. The concentration of this standard is ~20 µg/ mL RPA 717879.

3. a. Using a class "A" volumetric pipette, transfer 10 mL of the mixed standard (step III.A.2a.) to a 100-mL volumetric flask. Dilute to mark with acetonitrile. Cap and mix by inversion. The concentration of this mixed standard is ~2 µg/mL RPA 407213, RPA 408056, RPA 410914, and RPA 406012.

   b. Using a class "A" volumetric pipette, transfer 10 mL of the standard (step III.A.2b.) to a 100-mL volumetric flask. Dilute to mark with acetonitrile. Cap and mix by inversion. The concentration of this standard is ~2 µg/mL RPA 717879.

4. a. Using a class "A" volumetric pipette, transfer 1 mL of the mixed standard (step III.A.3a.) to a 100-mL volumetric flask. Dilute to mark with 20% acetonitrile in water. Cap and mix by inversion. The concentration of this mixed standard is ~0.02 µg/mL RPA 407213, RPA 408056, RPA 410914, and RPA 406012.

   b. Using a class "A" volumetric pipette, transfer 5 mL of the standard (step III.A.3b.) to a 100-mL volumetric flask. Dilute to mark with 20% acetonitrile in water. Cap and mix by inversion. The concentration of this mixed standard is ~0.1 µg/mL RPA 717879.

5. a. For the preparation of calibration standards of RPA 407213, RPA 408056, RPA 410914, and RPA 406012 perform the following dilutions using the ~0.02 µg/mL mixed standard (step III.A.4a) :

mL of the ~0.02µg/mL mixed standard	Added to mL 20% acetonitrile in water	Concentration µg/mL
1.5	100	0.0003
3.0	100	0.0006
5.0	100	0.001
10	100	0.002
15	100	0.003

b. For the preparation of calibration standards of RPA 717879 perform the following dilutions using the ~0.1 µg/mL standard (step III.A.4b) :

mL of the ~0.1 µg/mL standard	Added to mL 20% acetonitrile in water	Concentration µg/mL
4.0	100	0.004
6.0	100	0.006
10	100	0.010
15	100	0.015

B. Stability

1. To evaluate the stability, the following formula has been used :

   percent stability = [1-(old std. soln. / new std. soln.)] x 100

   The old standard solution should give detector responses within 10% of those of the new standard solution in order for the given standard solution to be considered stable under the storage conditions.

2. Stock solutions: Each product prepared in acetonitrile and stored at 4°C ± 3°C was stable for up to 4 months[1].

3. The storage of solutions of less than 20 µg/mL should be no longer than 30 days. These solutions may be replaced earlier if deemed necessary.

IV. METHOD PROCEDURES

A. General Notes

A1. The "♦"symbol indicates an optional stopping point after completing the indicated step. Samples may be stored overnight in a refrigerator (at or below 10°C).

A2. The "~" symbol indicates 'approximately.'

A3. Conditioning of the cartridges in step B13 can be started earlier and does not have to be done after the completion of steps B1-B11. However, the cartridges should be used the day of conditioning.

A4. *A flow rate of ~2 mL/min is critical and should be maintained throughout the conditioning and elution process (unless otherwise specified). A faster flow rate will result in low recoveries. Cartridges should not be allowed to run dry.*

B. Soil

(Analysis for RPA 407213, RPA 408056, RPA 717879, RPA 410914, and RPA 406012)

♦ B1. Weigh ~20 g of sample into a 250 mL Nalgene® bottle. The sample may be stored in a refrigerator until needed.

B2. For recoveries, fortify the sample with the appropriate standard solution.

B3. Add ~100 mL of 75% acetone in water, add a stir bar and place on a magnetic stirrer for ~30 minutes.

B4. Remove the stir bar. Centrifuge at ~4000 rpm for ~10 minutes.

B5. Decant and vacuum filter the supernatant through a 541 filter (top) and two GF/C filters into a tared 500mL evaporation flask. Wet filter paper with water prior to filtering in order to hold it down.

B6. Add ~100 mL of 75% acetone in water to the sample bottle, add the stir bar and place on a magnetic stirrer for ~30 minutes.

B7. Remove the stir bar. Centrifuge at ~4000 rpm for ~10 minutes.

B8. Decant and vacuum filter the supernatant through a 541 filter (top) and two GF/C filters into a tared 500mL evaporation flask. Wet filter paper with water prior to filtering in order to hold it down.

♦ B9. Scrape the soil from the bottom of the Nalgene® bottle with a spatula and transfer it to the filter. Wash the bottle twice with 15mL of 75% acetone in water and transfer it to the filter.

B10. Evaporate the extract to ~5 mL using a rotary evaporator with a <40°C water bath, then hold under a stream of Nitrogen for ~1 min. and then add 5 mL of water and sonicate for ~30 seconds. *The acetone must be removed completely. This is a critical step.*

B11. Transfer the extract to a 50mL centrifuge tube and centrifuge at ~2500 rpm for 10 minutes.

B12. Immediately set-up an HR-P cartridge and a stopcock on a purification system connected to a vacuum. Insert a plug of ~0.25 g of glass wool into the cartridge. Place a cartridge adaptor and a reservoir on top of the cartridge.

B13. Condition the cartridge with ~15 ml of acetonitrile followed by ~15 ml of 50% methanol in water. (~1 drop/2 sec, ~2 mL/min. Do not allow the cartridge to dry).

B14. Decant the extract onto the cartridge (~1 drop/2 sec, ~2mL/min). Elute (~1 drop/2 sec, ~2 mL/min. Do not allow the cartridge to dry) and discard the effluent.

B15. Add ~15mL of 50% methanol in water to the evaporation flask as a rinse and transfer the contents to the same centrifuge tube. Vortex or mix the centrifuge tube thoroughly. Centrifuge at ~2500 rpm for 10 minutes and decant onto the cartridge. Elute (~1 drop/2 sec, ~2 mL/min. Do not allow the cartridge to dry) and discard the effluent.

♦ B16. Set-up a 125 ml evaporation flask. Add ~30 mL of acetonitrile to the evaporation flask as a rinse and transfer the contents onto the cartridge. Elute the compounds of interest from the cartridge (~1 drop/2 sec, ~2 mL/min. Do not allow the cartridge to dry).

B17. Rotary evaporate to nearly dryness using a water bath temperature of ~40°C and a vacuum of ~27 in. Hg. As solvent evaporates from the flask, adjust the level of the flask in the water bath so that only the solution is being heated.

♦ B18. Add an appropriate amount of 20% acetonitrile in water, 0.2% acetic acid to each flask to dissolve the residues and sonicate for ~ 2 minutes. Suggested dilution volumes are 20mL for analysis of RPA 717879 at the proposed LOQ of 10ppb. Filter a portion of the 20mL extract to be used for the analysis of RPA 717879 only through a Nylon Acrodisc® 13mm, 0.45 µm syringe filter. To analyze for the other compounds, a 1mL aliquot of the unfiltered 20mL extract is transferred to a 10mL volumetric flask and diluted to the mark with 20% acetonitrile in water, 0.2% acetic acid. The samples are ready for LC/MS/MS analysis.

V. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY-MASS SPECTROMETRY (LC/MS/MS)

A. Conditions

Instrument used: Perkin Elmer Sciex API III+ LC/MS/MS system
Hitachi L6200 HPLC pump
PE Turbo IonSpray Electrospray Interface.
Hitachi AS2000 autosampler

Ionization: Electrospray (TurboIonSpray), positive ion mode

Curtain gas flow: Nitrogen at ~1.2 L/min

Nebulizer pressure: 55 psi

Turbo IonSpray Settings: Heated air at ~4.75 L/min, 500°C

MS Mode: MS/MS with multiple reaction monitoring (MRM)

IonSpray / Orifice voltage: 5500V / 65 V

Collision gas: Argon at approximately 275 x 10^{13} atoms/cm^2

Collision energy (R2-R0): 13V - 30V= -17V

Mass Transitions:

717879:	191/120
408056:	221/120
407213:	312/236
406012:	357/120
410914:	357/120

Column: Alltech Altima C18 5u, 100x4.6mm

Mobile phase flow rate: 1.0 mL/min split to ~200µL/min

Mobile phase composition:

A=acetonitrile
B=1.0% acetic acid in HPLC grade water
Gradient program (dwell volume < ~1.5mL)

Time (min)	%A	%B
0.0	30.0	(100-A)
3.0	100.0	
3.5	100.0	
3.6	30.0	

~11 min between injections

Injection volume: 75 µL

B. Alternate Conditions

Instrument used: Perkin Elmer Sciex API 300 LC/MS/MS system
Hewlett Packard HP1100 QuatPump and Vacuum Degasser
PE Turbo IonSpray Electrospray Interface.
Hewlett Packard HP1100 Autosampler

Ionization: Electrospray (TurboIonSpray), positive ion mode

Curtain gas flow: Nitrogen at ~1.08 L/min

Nebulizer gas flow: Nitrogen at ~1.31 L/min

Turbo IonSpray Settings: Heated Nitrogen at ~7 L/min, 450°C

MS Mode: MS/MS with multiple reaction monitoring (MRM)

IonSpray / Orifice voltage: 5200V / 30 V

Collision gas: Nitrogen at approximately 0.82 L/min

Collision energy (R2-R0): 25V - 9V= -16V

Mass Transitions:

717879:	191/120
408056:	221/120
407213:	312/236
406012:	357/120
410914:	357/120

Column: Alltech Alltima C18 5u, 100x4.6mm
Guard Column : Alltech Alltima C18 5 micron

Mobile phase flow rate: 1.0 mL/min split to ~200µL/min
Mobile phase composition:

A=acetonitrile
B=0.2% acetic acid in HPLC grade water

Gradient program

Time (min)	%A	%B
0.0	30.0	(100-A)
1.0	30.0	
3.0	100.0	
4.0	100.0	
8.0	30.0	
9.0	STOP	

~11 min between injections

Injection volume: 100 µL

Note the indicated LC/MS/MS parameters are guidelines and should be optimized for the instrument and column actually used. Instrument parameters and mobile phase compositions may be adjusted to improve separation from interfering peaks.

APPROXIMATE RETENTION TIMES

RPA 717879	2.25 minutes
RPA 408056	3.14 minutes
RPA 406012	5.16 minutes
RPA 407213	5.21 minutes
RPA 410914	5.27 minutes

Retention times may vary from those presented above.

Example chromatograms are attached (see section X). Note that the retention times may vary from system to system.

C. Performance Criteria

First criterion:
Run a standard solution corresponding to a level at or below the estimated LOQ and obtain a signal to noise ratio of at least 9:1.

If this criterion cannot be met, optimize instrument operating parameters or change instrument method parameters such as split ratio or injection size until a signal to noise ratio of 9:1 is obtained.

If this criterion still cannot be met by changing operating parameters, run higher level standards until a signal to noise ratio of 9:1 is obtained. This will require adjusting the method final sample dilution such that this standard level corresponds to the required LOQ.

Second criterion:
Run a set of standards of four or more concentration levels, from at or below the LOQ, up to the highest concentration level to be included in the analysis. Generate a calibration curve for each analyte and obtain a linear regression with a correlation coefficient of at least 0.90 for each analyte. If this criterion is met, the samples may be run with standards interspersed. Do not use any sample run data if the combined regression for standards run immediately before, during and after the samples do not meet this criterion.

Note:
To stabilize the response of the instrument, it has been found useful to run at least one standard and three or more sample or untreated control solutions as "wake up" runs before the actual runs to be used in calculations are commenced.

VI. CALCULATIONS

Linear regression should be used to generate calibration curves for RPA 407213, RPA 717879, RPA 408056, RPA 406012 and RPA 410914. After the instrument performance criteria are met, a minimum of four standards over a range of concentration levels should be included with a set of samples. Standards should be interspersed with samples to compensate for any minor change in instrument response. Samples should be diluted such that any peak areas or heights are within the area or height range between the lowest and highest standards injected.

Linear regression coefficients should be calculated on standard concentration (ng/mL) versus peak area or height. The data from the analytical standards should then be fit to the linear model,

$$Y = A + BX.$$

The equation to be used to estimate the residues in the samples is:

$$E = \frac{(Y - A)}{B} \cdot \frac{C}{D}$$

where: Y = response of analyte of interest (peak area or height)
A = intercept from linear regression analysis (peak area or height)
B = slope from linear regression analysis (response per concentration)
C = final sample volume (mL)
D = starting weight in grams of sample in final volume (g)
E = concentration of analyte in sample in parts per billion (ppb or ng/mL)

VII. SAFETY

All available appropriate Material Safety Data Sheets should be available to the study personnel during the conduct of the study. General laboratory safety precautions should be taken. This method does not present any specific risks.

VIII. REFERENCES

1. "RPA 407213 and its metabolites: Analytical method for the determination of residues in soil" AR 138-96 F. Martial, B. Simonin, C. Venet, June 27, 1997.