Method of Analysis for Isoxaflutole and Its Metabolites in Soil

I. INTRODUCTION

A. Scope

This method describes the procedure for determining the residues of isoxaflutole and its metabolites, RPA 202248 and RPA 203328, in soil.

B. Principle

Residues of isoxaflutole, RPA 202248 and RPA 203328 are extracted from soil by shaking the sample in an acetonitrile: 0.8% formic acid solution for fifteen minutes. The mixture is centrifuged and the supernatant is diluted to the desired concentration for analysis by LC/MS/MS using a C_8 HPLC column.

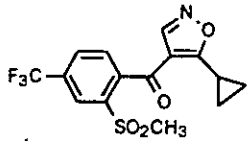
Quantification of results is based on internal standard area ratios for each analyte. The ^{13}C isotopically labeled internal standard differs from the ^{12}C analytes by six mass units.

C. Method Limits

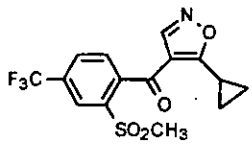
The method detection limit (MDL) and limit of quantification (LOQ) for isoxaflutole, RPA 202248 and RPA 203328 in soil will be established for this method during validation.

D. Chemical Structures

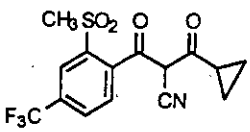
RPA 201772 (^{12}C)
m.w. 359.35



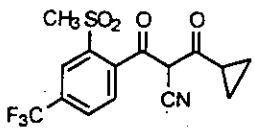
RPA 201772 ($^{13}C_6$)
m.w. 365.35



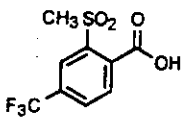
RPA 202248 (^{12}C)
m.w. 359.3



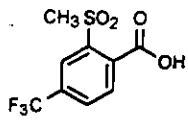
RPA 202248 ($^{13}C_6$)
m.w. 365.3



RPA 203328 (^{12}C)
m.w. 268.21



RPA 203328 ($^{13}C_6$)
m.w. 274.21



II. MATERIALS

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

A. Reagents/Solvents

Acetic Acid GR (EM Science, Cat. No. AX0073-13)

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

Formic Acid Suprapur (EM Science, Cat. No. 11670-1)

Water (HPLC grade) (VWR WX004-1)

Calibration Buffer Solutions pH 4.0 and 7.0
(Orion, Cat. No. 910104 and 910107)

B. Equipment and Supplies

All re-usable glassware should be baked in a muffle oven at ~450 °C for at least 2 hours to remove any possible contamination before use.

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

Bottles, amber, 4 oz. (Qorpak)

Spatula

Disposable pipettes

Graduated cylinders

Column, HPLC, Columbus C8, 50 mm x 2.0 mm id, 5µm
(Phenomenex, Cat. No. 00D-4187-E0, *no substitute*)

Mechanical Shaker

Nalgene PP 125 mL and 250 mL wide mouth bottles
(VWR # 2105-0004 and 2105-0008)

Magnetic stirrer and stir bar

Centrifuge (2500 rpm capacity)

Acrodisc CR PTFE syringe filters (0.2 µm)

Pipette bulb

Precolumn HPLC Filter, Ultra Low Dead Volume, 0.5 µm frit

(Upchurch, A-318)

Solvent jugs, 4 L brown glass

Volumetric flasks (Class A)

Volumetric pipettes (Class A)

pH meter

Eppendorf pipettors

16 X 100 mm disposable culture tubes (VWR # 60825-425)

Vial, clear, 1.5 mL; cap, open top; septa, split
(Sun, 200-250; 200-292, 500-870)

C. Solutions

The following is a list of the solutions used in the analysis 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 ~0.8% Formic Acid in Water, pH 2.1
   Calibrate pH meter prior to preparing the solution. Place a 4 L brown glass jug on a stir plate and add stir bar. Fill jug with ~4.0 L of H_2O. Add ~28 mL of formic acid. Put the pH meter probe into the water and measure the pH of the water while it is stirring. Using a disposable pipette, add formic acid drop-wise until a pH of 2.10 ± 0.05 is reached.

2. 80:20 Solution of Acetonitrile:~0.8% Formic Acid in Water, pH 2.1
   Using graduated cylinders, combine 200 mL of ~0.8% formic acid (solution 1) and 800 mL of acetonitrile in a 4 L brown glass solvent jug that is clean and dry or a jug which was previously used for this solution. Repeat until the desired quantity has been made.

3. 90:10 Solution of ~0.8% Formic Acid in Water, pH 2.1:Acetonitrile

Using graduated cylinders, combine 900 mL of ~0.8% formic acid (solution 1) and 100 mL of acetonitrile in a 4 L brown glass solvent jug that is clean and dry or a jug which was previously used for this solution. Repeat until the desired quantity has been made.

4. Solution of ~1.5% Acetic Acid in Water

Pour ~950 mL of water into a 1 L graduated cylinder. Using a volumetric pipette, add 15 mL of acetic acid. Make up to 1 liter with water. Transfer to a 4 L brown glass jug. Mix by shaking. Repeat until the desired quantity has been made.

D. Analytical Standards

1. Common name/alias: Isoxaflutole, RPA 201772
4-(2-methanesulphonyl-4-trifluoromethylbenzoyl-5-cyclopropylisoxazole

Chemical name: Methanone, (5-cyclopropyl-4-isoxazolyl) [2-(methylsulfonyl)-4-(trifluoromethyl)phenyl] (CAS No. 141112-29-0)

Solubility [1]:

acetone:	29.3(g/100ml)
acetonitrile:	23.3
hexane:	0.010
methanol:	1.38
drinking water (pH 5.5):	0.00062

2. Common name/alias: Isoxaflutole, RPA 201772 **($^{13}C_6$) labeled**
4-(2-methanesulphonyl-4-trifluoromethylbenzoyl-$^{13}C_6$)-5-cyclopropylisoxazole

Chemical name: Methanone, (5-cyclopropyl-4-isoxazolyl) [2-(methylsulfonyl)-4-(trifluoromethyl)phenyl-$^{13}C_6$]

3. Common name/alias: RPA 202248
2-cyano-3-cyclopropyl-1-(2-methylsulphonyl-4-trifluoromethylphenyl)-propan-1,3-dione

Chemical name: Benzenepropanenitrile,α-(cyclopropylcarbonyl)

-2-(methylsulfonyl)β-oxo-4-(trifluoromethyl)
(CAS No. 143701-75-1)

4. Common name/alias: RPA 202248 **($^{13}C_6$) labeled**
2-cyano-3-cyclopropyl-1-(2-methylsulphonyl-4-trifluoromethylphenyl-$^{13}C_6$)-propan-1,3-dione

Chemical name: Benzene-$^{13}C_6$-propanenitrile,α-(cyclopropylcarbonyl)-2-(methylsulfonyl)β-oxo-4-(trifluoromethyl)

5. Common name/alias: RPA 203328
2-methanesulphonyl-4-trifluoromethylbenzoic acid

Chemical name: Benzoic acid, 2-(methylsulfonyl)-4-(trifluoromethyl)
(CAS No. 142994-06-7)

6. Common name/alias: RPA 203328 **($^{13}C_6$) labeled**
2-methanesulphonyl-4-trifluoromethylbenzoic acid-$^{13}C_6$

Chemical name: Benzoic acid-$^{13}C_6$, 2-(methylsulfonyl)-4-(trifluoromethyl)

III. FORTIFICATION AND CALIBRATION STANDARD SOLUTIONS 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.

All re-useable glassware should be baked in a muffle oven at ~450°C for at least 2 hours to remove any possible contamination before use.

From this point on, the term **fortification** standard will refer to the ^{12}C compound.

From this point on, the term **internal** standard will refer to the ^{13}C compound.

A. Fortification Standards

1. Weigh ~0.1000 g (corrected for purity) each of isoxaflutole, RPA 202248 and RPA 203328 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 ~1000 µg/mL.

2. Transfer 10 mL each of the ~1000 µg/mL of isoxaflutole, RPA 202248 and RPA 203328, via volumetric class "A" pipettes, to one 100 mL volumetric flask. Dilute to mark with a 90:10 solution of ~0.8% formic acid in water: acetonitrile, pH 2.1. Cap and mix by inversion. The concentration of this mixed standard is ~100 µg/mL RPA 203328, isoxaflutole and RPA 202248.

3. Using a class "A" volumetric pipette, transfer 1 mL of the mixed standard (step III.A.2.) to a 100-mL volumetric flask. Dilute to mark with a 90:10 solution of ~0.8% formic acid in water: acetonitrile, pH 2.1. Cap and mix by inversion. The concentration of this mixed standard is ~1 µg/mL RPA 203328, isoxaflutole and RPA 202248.

4. Using a class "A" volumetric pipette, transfer 10 mL of the mixed standard (step III.A.3.) to a 100-mL volumetric flask. Dilute to mark with a 90:10 solution of ~0.8% formic acid in water: acetonitrile, pH 2.1. Cap and mix by inversion. The concentration of this mixed standard is ~0.1 µg/mL RPA 203328, isoxaflutole and RPA 202248.

5. Using a class "A" volumetric pipette, transfer 10 mL of the mixed standard (step III.A.4.) to a 100-mL volumetric flask. Dilute to mark with a 90:10 solution of ~0.8% formic acid in water: acetonitrile, pH 2.1. Cap and mix by inversion. The concentration of this mixed standard is ~10 ng/mL RPA 203328, isoxaflutole and RPA 202248.

6. Using a class "A" volumetric pipette, transfer 10 mL of the mixed standard (step III.A.5.) to a 100-mL volumetric flask. Dilute to mark with a 90:10 solution of ~0.8% formic acid in water: acetonitrile, pH 2.1. Cap and mix by inversion. The concentration of this mixed standard is ~1 ng/mL RPA 203328, isoxaflutole and RPA 202248.

B. $^{13}C_6$ Labeled Internal Standards

1. Weigh ~0.0100 g (corrected for purity) each of $^{13}C_6$ isoxaflutole, $^{13}C_6$ RPA 202248 and $^{13}C_6$ RPA 203328 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 ~100 µg/mL.

2. Transfer 10 mL each of the ~100 µg/mL of $^{13}C_6$ isoxaflutole, $^{13}C_6$ RPA 202248 and $^{13}C_6$ RPA 203328, via volumetric class "A" pipettes, to one 100 mL volumetric flask. Dilute to mark with a 90:10 solution of ~0.8% formic acid in water: acetonitrile, pH 2.1. Cap and mix by inversion. The concentration of this mixed standard is ~10 µg/mL $^{13}C_6$ RPA 203328, $^{13}C_6$ isoxaflutole and $^{13}C_6$ RPA 202248.

3. Using a class "A" volumetric pipette, transfer 10 mL of the mixed standard (step III.B.2.) to a 100-mL volumetric flask. Dilute to mark with a 90:10 solution of ~0.8% formic acid in water: acetonitrile, pH 2.1. Cap and mix by inversion. The concentration of this mixed standard is ~1 µg/mL $^{13}C_6$ RPA 203328, $^{13}C_6$ isoxaflutole and $^{13}C_6$ RPA 202248.

4. Using a class "A" volumetric pipette, transfer 10 mL of the mixed standard (step III.B.3.) to a 100-mL volumetric flask. Dilute to mark with a 90:10 solution of ~0.8% formic acid in water: acetonitrile, pH 2.1. Cap and mix by inversion. The concentration of this mixed standard is ~0.1 µg/mL $^{13}C_6$ RPA 203328, $^{13}C_6$ isoxaflutole and $^{13}C_6$ RPA 202248.

5. Using a class "A" volumetric pipette, transfer 10 mL of the mixed standard (step III.B.4.) to a 100-mL volumetric flask. Dilute to mark with a 90:10 solution of ~0.8% formic acid in water: acetonitrile, pH 2.1. Cap and mix by inversion. The concentration of this mixed standard is ~10 ng/mL $^{13}C_6$ RPA 203328, $^{13}C_6$ isoxaflutole and $^{13}C_6$ RPA 202248.

6. Using a class "A" volumetric pipette, transfer 10 mL of the mixed standard (step III.B.5.) to a 100-mL volumetric flask. Dilute to mark with a 90:10 solution of ~0.8% formic acid in water: acetonitrile, pH 2.1. Cap and mix by inversion. The concentration of this mixed standard is ~1 ng/mL $^{13}C_6$ RPA 203328, $^{13}C_6$ isoxaflutole and $^{13}C_6$ RPA 202248.

C. Calibration Standards

1. ~ 0.1 ng/mL Mixed ^{12}C Std with 0.1 ng/mL Mixed ^{13}C Internal Std
   Using a class "A" volumetric pipette, transfer 10 mL of the ~1.0 ng/mL mixed **internal** standard (step III.B.6.) and 10 mL of the ~1.0 ng/mL mixed **fortification** standard (step III.A.6.) to a 100-mL volumetric flask. Dilute to mark with a 90:10 solution of ~0.8% formic acid in water: acetonitrile, pH 2.1. Cap and mix by inversion.

2. ~ 0.08 ng/mL Mixed ^{12}C Std with 0.1 ng/mL Mixed ^{13}C Internal Std
   Using a class "A" volumetric pipette, transfer 10 mL of the ~1.0 ng/mL mixed **internal** standard (step III.B.6.) and 8 mL of the ~1.0 ng/mL mixed **fortification** standard (step III.A.6.) to a 100-mL volumetric flask. Dilute to mark with a 90:10 solution of ~0.8% formic acid in water: acetonitrile, pH 2.1. Cap and mix by inversion.

3. ~ 0.05 ng/mL Mixed ^{12}C Std with 0.1 ng/mL Mixed ^{13}C Internal Std
   Using a class "A" volumetric pipette, transfer 10 mL of the ~1.0 ng/mL mixed **internal** standard (step III.B.6.) and 5 mL of the ~1.0 ng/mL mixed **fortification** standard (step III.A.6.) to a 100-mL volumetric flask. Dilute to mark with a 90:10 solution of ~0.8% formic acid in water: acetonitrile, pH 2.1. Cap and mix by inversion.

4. ~ 0.02 ng/mL Mixed ^{12}C Std with 0.1 ng/mL Mixed ^{13}C Internal Std
   Using a class "A" volumetric pipette, transfer 10 mL of the ~1.0 ng/mL mixed **internal** standard (step III.B.6.) and 2 mL of the ~1.0 ng/mL mixed **fortification** standard (step III.A.6.) to a 100-mL volumetric flask. Dilute to mark with a 90:10 solution of ~0.8% formic acid in water: acetonitrile, pH 2.1. Cap and mix by inversion.

5. ~ 0.01 ng/mL Mixed ^{12}C Std with 0.1 ng/mL Mixed ^{13}C Internal Std
   Using a class "A" volumetric pipette, transfer 10 mL of the ~1.0 ng/mL mixed **internal** standard (step III.B.6.) and 1 mL of the ~1.0 ng/mL mixed **fortification** standard (step III.A.6.) to a 100-mL volumetric flask. Dilute to mark with a 90:10 solution of ~0.8% formic acid in water: acetonitrile, pH 2.1. Cap and mix by inversion.

D. 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 5.5 months[2].

3. 100 µg/L standard solutions: A solution of isoxaflutole , RPA 202248, and RPA 203328 prepared in 80:20 water: acetonitrile was stable for up to 5.5 months [2-4].

4. 100 µg/L standard solutions: A solution of isoxaflutole , RPA 202248, and RPA 203328 prepared in 90:10 ~0.8% formic acid: acetonitrile (pH 2.1) was stable for at least one month.

IV. METHOD PROCEDURES

A. Extraction and Clean-up

A1. Weigh 50 g (+/-0.1g) of sample into a 125 mL Nalgene® bottle. The sample may be stored in a freezer until needed.

A2. For recoveries, fortify the sample with the appropriate ^{12}C and ^{13}C standard solutions to the desired concentration. Mix the soil to disperse the analytes and allow the samples to equilibrate at room temperature for 15-30 minutes.

A3. For UTC and Unknown samples, fortify the sample, with the appropriate ^{13}C standard solution, to the desired concentration. Mix the soil to disperse the analytes and allow the samples to equilibrate at room temperature for 15-30 minutes.

A4. Add ~75 mL of 80:20 acetonitrile: 0.8% formic acid and mix on a mechanical shaker for ~15 minutes.

A5. Centrifuge the shaken sample at ~2500 rpm for ~ five minutes.

A6. Decant the supernatant into a 250 mL Nalgene bottle.

A7. Repeat steps A4-A6 one more time. *Note: Disperse the pellet prior to the second mechanical mixing.* Combine the two supernatants and mix.

A8. Remove 2 mL of the extract and combine it with 4 mL of the 0.8% formic acid solution and mix. *Note: The organic to aqueous ratio for the final solution will be ~26:74. It was determined that peak shape begins to deteriorate as the organic ratio rises about 30 %.*

A9. Filter the diluted solution through a syringe filter into an HPLC vial after sending the first ~0.5-1 mL to waste.

A10. Sample is ready for LC/MS/MS analysis.

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

A. Conditions

Instrument used:	Perkin Elmer Sciex API 3000 LC/MS/MS System PE Sciex Turbo Ion Spray Electrospray Interface. Shimadzu LC-10AD VP HPLC Pumps (2) with 250µL High Pressure Mixer and SCL-10A VP Pump Controller Perkin Elmer Series 200 Autosampler
Ionization and MS Mode:	Electrospray (TurboIonSpray) - negative ion mode MS/MS with multiple reaction monitoring (MRM)
IonSpray / Orifice Voltage:	-4800V / -59V
Nebulizer Setting:	15 (Air)
Curtain Gas Setting:	9 (Nitrogen)
Turbo IonSpray Settings:	Heated air at ~8.5 L/min, 500°C
Collision Gas Setting:	8 (Nitrogen)

Collision Energy (R02-Q0): (36-10)V = 26V
(*See data for complete list of instrument dependent state file parameters*)

Mass Transitions (Dwell times in milli seconds):

Period 1:		
RPA203328:	267/159	(375 ms)
$^{13}C_6$ RPA203328:	273/165	(275)
Period 2:		
RPA202248 and IFT:	358/79	(375)
$^{13}C_6$ RPA202248 and IFT:	364/79	(275)

Column: Phenomenex, Columbus C8, 2.0 x 50 mm, 5µm particle size, 100A pore size

Mobile phase flow rate: 0.400 mL/min no split

Mobile phase: 48% Acetonitrile / 52% (1.5% Acetic acid in HPLC water)

Injection volume: 50 µL

Retention times: See chromatograms and data reports

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 203328	1.15 minutes
RPA 201772	2.25 minutes
RPA 202248	3.40 minutes

Retention times may vary from those presented above.

B. 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 can not 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 can not 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 201772, RPA 202248, and RPA 203328. The regression will be plotted as concentration vs. area ratio for each standard level. The area ratio is calculated by dividing the area of the ^{12}C analyte by the area of the corresponding ^{13}C internal standard analyte. 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 ^{12}C peak areas or heights are within the area or height range between the lowest and highest ^{12}C 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 determine the residues in the samples is:

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

where: Y = response of analyte of interest (area ratio)
A = intercept from linear regression analysis
B = slope from linear regression analysis (response per concentration)
C = final sample volume (mL)
D = sample weight (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. "Isoxaflutole and/or metabolites: an analytical method for the determination of residues in drinking water" AR 112-95 I. Le Gren, July 27, 1995.
2. "Isoxaflutole and/or metabolites: Analytical method for the determination of residues in soil" AR 106-95(E) I. Le Gren, Jan 17, 1995.
3. "Isoxaflutole and/or metabolites: Analytical method for the determination of residues in animal products" AR 109-95(E) I. Le Gren, April 12, 1995.