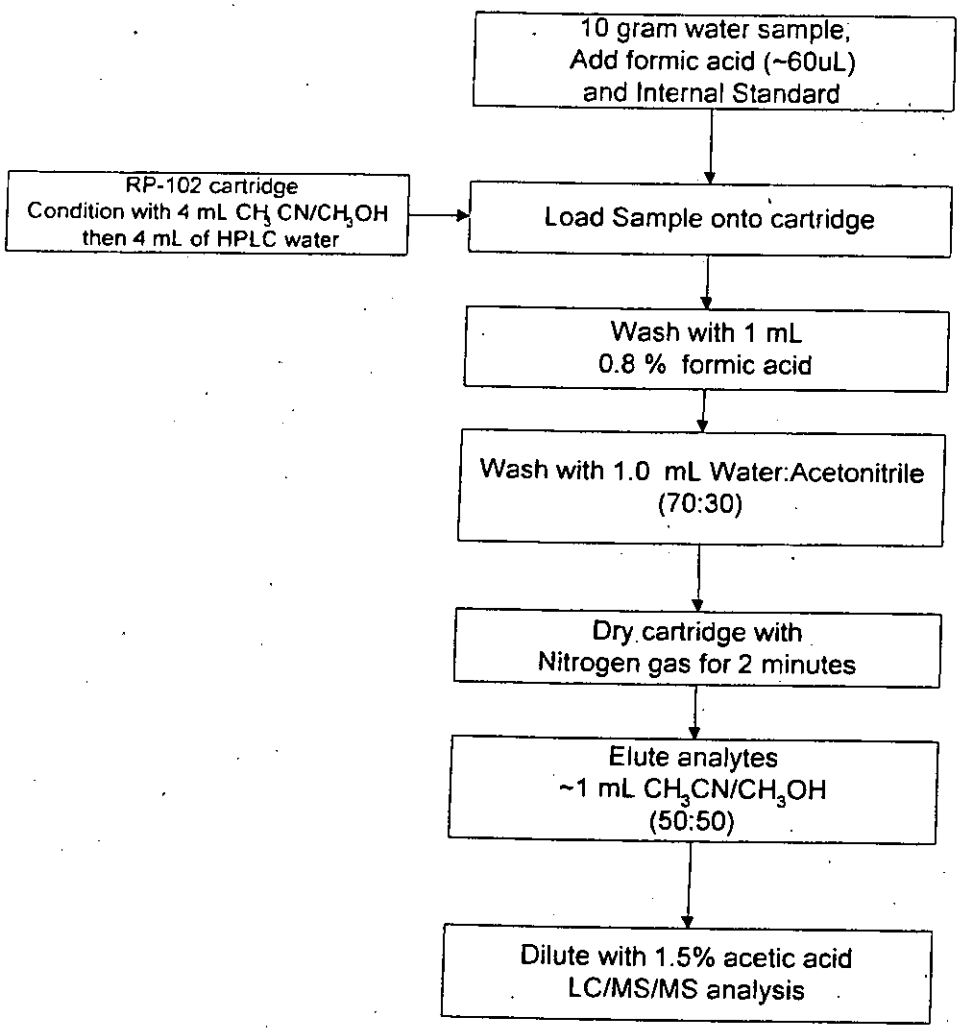
Summary Flowchart of Analytical Method



Method of Analysis For the Quantification of Isoxaflutole and Its Metabolites in Water Using Isotopically Labeled Internal Standards - Revision 99.2

I. INTRODUCTION

A. Scope

This method sets forth the procedure for determining the residues of isoxaflutole and its metabolites RPA 202248 and RPA 203328 in ground and surface water.

B. Principle

An analytical method is described for the determination of residues of isoxaflutole and its metabolites (RPA 202248 and RPA 203328) in ground and surface water. Residues of isoxaflutole, RPA 202248 and RPA 203328 are extracted from water using a RP-102 resin cartridge, then removed with acetonitrile:methanol.

All residue analysis is accomplished by LC-MS-MS on a C8 column. Quantification of results is based on a comparison of the ratio of analyte response to internal standard response versus analyte response to internal standard response of known standards.

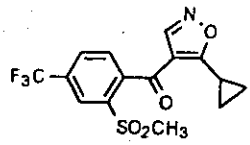
The use of isotopically labeled internal standards in the method causes sample recoveries to be relative and not absolute. The term recovery is better expressed as 'spiked sample accuracy' and as such reflects the accuracy of the method to correctly determine the level of analytes in a given sample. An estimate of the absolute recovery is obtained by comparison of the response to the internal standards in extracts of fortified samples with the response of the internal standard in calibration solutions.

C. Method Limits

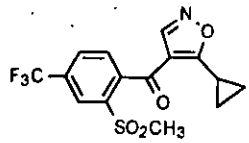
The limit of detection (LOD) was determined to be 1 parts per trillion (ppt, pg/g), for isoxaflutole, 1 ppt for RPA 202248, and 3 ppt for RPA 203328. The method has a validated LOQ of 10 ppt for isoxaflutole, RPA 202248, and RPA 203328.

D. Chemical Structures

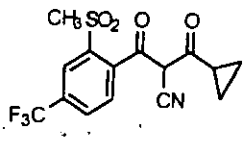
RPA 201772 ($^{12}C_6$)
m.w. 359.35



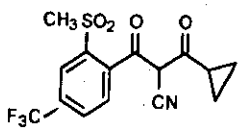
RPA 201772 ($^{13}C_6$)
m.w. 365.35



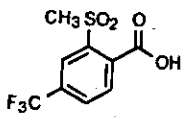
RPA 202248($^{12}C_6$)
m.w. 359.3



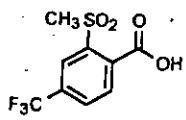
RPA 202248($^{13}C_6$)
m.w. 365.3



RPA 203328($^{12}C_6$)
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)
Methanol Omni-Solv	(EM Science, Cat. No. MX0488-1)
Water	(HPLC grade)

B. Equipment and Supplies

Adapters (plastic), 1, 3 and 6 mL (Varian, 1213-1001)
(manual procedure)

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)

Cartridges ,Spe-ed™ SPE, RP-102 Resin (200 mg/3mL)
(Applied Separations, Cat. No. 4208, *no substitute*)

Cartridge Adapters, SPE
(University Research Glass, Cat. No. URG-2440-SPECA)
(manual procedure)

Culture tube, disposable, 16 X 100mm (VWR, Cat No. 60825-425)
(automation procedure)

Disposable pipettes

Graduated cylinders

Column, HPLC, Columbus C8, 2.0 X 50mm, 5µm, 100A pore size
(Phenomenex, Cat. No. 00B-4187-B0, *no substitute*)

Pipette bulb

Precolumn HPLC Filter, Ultra Low Dead Volume, 0.5µm frit
(Upchurch, A-318)

Solvent jugs, 4 L brown glass

Stopcocks (plastic), Luer Lock (Varian, 1213-1005)
(manual procedure)

Volumetric flasks

Volumetric pipettes

Vials, chromatography, 2 or 4 ml, clear

Zymark Benchmate Series 1
(automation procedure)

C. Solutions

The following is a list of the solutions used in the analyses of ground and surface water. 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 solution. Place a 4 L brown glass jug onto a stir plate and add stir bar. Fill jug with ~3.0 L of H_2O. Add ~28mL 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 until a pH of 2.10 ± 0.02 is reached.

2. 90:10 Solution of ~0.8% Formic Acid in Water:Acetonitrile, pH 2.1
   Using a 1000 mL graduated cylinder, transfer 900 mL of a solution of ~0.8% formic acid in H_2O, pH=2.1 and 100 mL CH_3CN to 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. Solution of 70:30 Water:Acetonitrile
   Using a 1000 mL graduated cylinder, transfer 700 mL of H_2O and 300 mL CH_3CN to 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 50:50 Acetonitrile:Methanol
   Using a 1000 mL graduated cylinder, transfer 500 mL of CH_3OH and 500 mL CH_3CN to 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.

5. Solution of ~1.5% Acetic Acid in Water

Pour ~950 mL of water into a 1 liter graduated cylinder. Using a volumetric pipette, add 15 mL of acetic acid. Make up to one 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

Common name/alias: Isoxaflutole, RPA 201772
5-cyclopropyl-4-(2-methylsulfonyl-4-trifluoromethylbenzoyl)isoxazole

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

Solubility [1]:

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

Common name/alias: Isoxaflutole, RPA 201772 ($^{13}C_6$) labeled

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

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

Chemical name: Benzoic acid, 2-(methylsulfonyl)-4-(trifluoromethyl)

(CAS No. 142994-06-7)

Common name/alias: RPA 203328 ($^{13}C_6$) labeled

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

Common name/alias: RPA 202248
2-cyclopropylcarbonyl-3-(2-methylsulphonyl-4-trifluoromethylphenyl)-3-oxopropanenitrile

Chemical name: Benzenepropanenitrile,α-(cyclopropylcarbonyl)-2-(methylsulfonyl)-β-oxo-4-(trifluoromethyl)
(CAS No. 143701-75-1)

Common name/alias: RPA 202248 ($^{13}C_6$) labeled

Chemical name: Benzene-$^{13}C_6$- propanenitrile,α-(cyclopropylcarbonyl)-2-(methylsulfonyl)-β-oxo-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 reusable glassware should be baked in a muffle oven at ~450 °C for at least 2 hours to remove possible contamination before use.

A. *Fortification Standards (non-*$^{13}C_6$ labeled)

1. Weigh ~0.1000g (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 H_2O:CH_3CN, 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 ~100 µg/mL 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 H_2O:CH_3CN, 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 ~1 µg/mL 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 H_2O:CH_3CN, pH 2.1. Cap and mix by inversion. The concentration of this mixed standard is ~0.10 µg/mL RPA 203328, isoxaflutole and RPA 202248.

5. Using a class "A" volumetric pipette, transfer 10 mL of the ~0.10 µg/mL 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 H_2O:CH_3CN, pH 2.1. Cap and mix by inversion. The concentration of this mixed standard is ~0.01 µg/mL (~10.0 ng/ml) RPA 203328, isoxaflutole and RPA 202248.

6. Using a class "A" volumetric pipette, transfer 10 mL of the ~0.01 µg/mL 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 H_2O:CH_3CN, pH 2.1. Cap and mix by inversion. The concentration of this mixed standard is ~0.001 µg/mL (~1.0 ng/ml) RPA 203328, isoxaflutole and RPA 202248.

B. *$^{13}C_6$ labeled Internal Standards*

1. Weigh ~0.0100g (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 a 90:10 solution of ~0.8% formic acid in H_2O:CH_3CN, pH 2.1. 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 H_2O:CH_3CN, 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 ~10 µg/mL $^{13}C_6$ 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 H_2O:CH_3CN, 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 ~1 µg/mL $^{13}C_6$ 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 H_2O:CH_3CN, 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 ~0.1 µg/mL $^{13}C_6$ 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 H_2O:CH_3CN, pH 2.1. Cap and mix by inversion. The concentration of this mixed standard is ~0.01 µg/mL (~10.0 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 ~0. µg/mL (~10.0 ηg/ml) $^{13}C_6$ 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 H_2O:CH_3CN, pH 2.1. Cap and mix by inversion. The concentration of this mixed standard is ~0.001 µg/mL (~1.0 ng/mL) $^{13}C_6$ RPA 203328, $^{13}C_6$ isoxaflutole and $^{13}C_6$ RPA 202248.

C. *Calibration Standards*

1. Using a class "A" volumetric pipette, transfer 10 mL of the ~1.0 ng/mL *fortification* mixed standard (step III.A.6) and 10 ml of the ~1.0 ng/mL $^{13}C_6$ labeled mixed standard (step III.B.6) to a 100-mL volumetric flask. Dilute to mark with a 90:10 solution of ~0.8% formic acid in H_2O:CH_3CN, pH 2.1. Cap and mix by inversion.
The concentration of this mixed standard is ~0.1 ng/mL RPA 203328, isoxaflutole and RPA 202248 and ~0.1 ng/mL $^{13}C_6$ RPA 203328, $^{13}C_6$ isoxaflutole and $^{13}C_6$ RPA 202248

2. Using a class "A" volumetric pipette, transfer 8 mL of the ~1.0 ng/mL *fortification* mixed standard (step III.A.6.) and 10 ml of the ~1.0

ng/mL $^{13}C_6$ labeled mixed standard (step III.B.6) to a 100-mL volumetric flask. Dilute to mark with a 90:10 solution of ~0.8% formic acid in H_2O:CH_3CN, pH 2.1. Cap and mix by inversion. The concentration of this mixed standard is ~0.08 ng/mL RPA 203328, isoxaflutole and RPA 202248 and ~0.1 ng/ml $^{13}C_6$ RPA 203328, $^{13}C_6$ isoxaflutole and $^{13}C_6$ RPA 202248

3. Using a class "A" volumetric pipette, transfer 5 mL of the ~1.0 ng/mL *fortification* mixed standard (step III.A.6.) and 10 ml of the ~1.0 ng/mL $^{13}C_6$ labeled mixed standard (step III.B.6) to a 100-mL volumetric flask. Dilute to mark with a 90:10 solution of ~0.8% formic acid in H_2O:CH_3CN, pH 2.1. Cap and mix by inversion. The concentration of this mixed standard is ~0.05 ng/mL RPA 203328, isoxaflutole and RPA 202248 and ~0.1 ng/mL $^{13}C_6$ RPA 203328, $^{13}C_6$ isoxaflutole and $^{13}C_6$ RPA 202248

4. Using a class "A" volumetric pipette, transfer 2 mL of the ~1.0 ng/mL *fortification* mixed standard (step III.A.6.) and 10 ml of the ~1.0 ng/mL $^{13}C_6$ labeled mixed standard (step III.B.6) to a 100-mL volumetric flask. Dilute to mark with a 90:10 solution of ~0.8% formic acid in H_2O:CH_3CN, pH 2.1. Cap and mix by inversion. The concentration of this mixed standard is ~0.02 ng/mL RPA 203328, isoxaflutole and RPA 202248 and ~0.1 ng/mL $^{13}C_6$ RPA 203328, $^{13}C_6$ isoxaflutole and $^{13}C_6$ RPA 202248

5. Using a class "A" volumetric pipette, transfer 1 mL of the ~1.0 ng/mL *fortification* mixed standard (step III.A.6) and 10 ml of the ~1.0 ng/mL $^{13}C_6$ labeled mixed standard (step III.B.6) to a 100-mL volumetric flask. Dilute to mark with a 90:10 solution of ~0.8% formic acid in H_2O:CH_3CN, pH 2.1. Cap and mix by inversion. The concentration of this mixed standard is ~0.01 ng/mL RPA 203328, isoxaflutole and RPA 202248 and ~0.1 ng/mL $^{13}C_6$ RPA 203328, $^{13}C_6$ isoxaflutole and $^{13}C_6$ RPA 202248

6. *Additional calibration standards.*
Higher concentration standards (0.1 ng/mL to 5 ng/mL) may be necessary for samples with residues above the previously described curve. The amount of internal standard should continue to be ~0.1 ng/ml for these higher concentrations.

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. General Notes

A1. 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 B5 can be started earlier and does not have to be done after the completion of steps C1-C3. However, the cartridges should be used the day of conditioning.

A4. Throughout the conditioning and elution process (unless otherwise specified) cartridges should not be allowed to run dry.

A5. The flow rate for loading the water sample on the cartridges (step B5) is faster than the conditioning and elution flow rate

A6. Samples that are frozen may be thawed out at ambient temperature or overnight in a refrigerator.

A7. The suggested volume used for fortification (both $^{12}C_6$ & $^{13}C_6$ standards) is ≤ 0.25 ml of standards per 10 gram sample. Enough internal standard needs to be fortified to insure that the final sample internal standard concentration is approximately equal to the concentration of internal standard in the calibration standards.

B. Reanalysis Criteria

(one or more of the following may apply)

B1. Individual samples should be reanalyzed if any or all of the internal standard analytes are recoveried at ≤ 40% of the average of the corresponding internal standard analyte from the standard curve. The standards used to determine the average should be from the same data set as the individual sample. (Because the final sample volume is not volumetrically measured, internal standard recovery is an estimate and not absolute.)

B2. Individual samples should be reanalyzed if the ratio of analyte response to internal standard response is larger than the ratio of analyte response to internal standard response of the highest calibration standard. For reanalysis a smaller sample size may be used. The smaller sample should be brought to a starting weight of ~10 gram with HPLC water. The internal standard fortification should be consistent with previous analysis. *[An alternative to reanalysis is the re-injection of high ratio samples using high level calibration standards that bracket the samples. (see section III.C.6)]*

C. Ground and Surface Waters

(Analysis for Isoxaflutole (RPA 201772), RPA 202248 and RPA 203328)

C1. Weigh ~10 g of sample into an appropriately sized container. Acidify the sample with ~60 µL of formic acid. The sample may be stored in a refrigerator until needed.

C2. <u>For untreated controls and samples</u>, fortify the sample with the appropriate amount of $^{13}C_6$ labeled internal standard. Immediately add ~60 µL of formic acid. Cap and mix. See example in step C3.

C3. For 'sample accuracy', fortify the sample with the appropriate standard solutions, both non-labeled and ^{13}C labeled standards. Immediately add ~60 µL of formic acid. Cap and mix. *Do not allow fortified samples to sit at room temperature for more than ~2 hours or low recoveries of isoxaflutole may be obtained.*

Example fortification: 10 gram water sample spiked with 0.1 ng of analyte and 0.25 ng of internal standard. Final volume of 2.5 ml gives a final concentration of 0.04 ng/ml analyte and 0.1 ng/ml internal standard. The internal standard concentration now mirrors that of the calibration standards.

C4. Immediately set-up a RP-102 cartridge (200 mg) on a purification system. A reservoir may need to be placed on top of the cartridge.

C5. Condition the cartridge with ~4 ml of 50:50 acetonitrile/methanol followed by ~4 ml of HPLC water. (~2 mL/min. Do not allow the cartridge to dry).

C6. Apply prepared sample to the cartridge (~1 drop/2 sec).

C7. Add ~1.0 mL of a solution of 0.8 % formic acid in water to the cartridge. (~1 drop/2 sec. Do not allow the cartridge to dry). Elute and discard the effluent.

C8. Add ~ 1.0 mL of a 30:70 solution of acetonitrile/water to the cartridge (~1 drop/2 sec. Do not allow the cartridge to dry). Elute and discard the effluent.

C9. Dry the cartridge for ~2 minutes. Vacuum or positive nitrogen pressure may be used to dry the cartridge. If the samples are prepared on a automated system, 10-30 psi nitrogen pressure can be used. If the samples are prepared on a vacuum manifold system, then ~20 inches of mercury vacuum could be used.

C10. Add ~1.0 mL of 50:50 acetonitrile/methanol to the cartridge. Apply positive pressure and push the solvent onto the cartridge. Take precautions to insure that no eluent is lost. *Positive pressure can be applied via a hand held nitrogen line.*

Manual method, vent the pressure and allow the cartridge to soak for 1-2 minutes. Reapply pressure and elute all solvent (~1drop/second) into an appropriately sized volumetric flask or chromatography vial

Automated method, the entire 1 mL of acetonitrile/methanol should be *slowly* (~ 1 minute) eluted through the cartridge. Collect the eluent in an appropriately sized volumetric flask or chromatography vial.

C11. Dilute with ~1.5% acetic acid in H_2O . Mix. Samples are ready for LC-MS-MS analysis. Suggested final dilution volumes are ~2.5 mL for samples containing expected residues near the LOQ level of 10ppt.

ZYMARK BENCHMATE (Automated Sample Preparation)

A. Conditions

Flow Rates:

Aspirate:	0.75 ml/sec
Dispense	1.00 ml/sec
Internal Standard	0.10 ml/sec
Mix	1.25 ml/sec
Air Push	0.25 ml/sec

SPE Parameters:

Condition flow:	0.15 ml/sec
Load flow	0.08 ml/sec
Rinse flow	0.15 ml/sec
Elute flow	0.02 ml/sec
Push delay	5 seconds
Air factor	1.5

Method Procedure:

Step 1:	Add internal standard
Step 2:	Mix by cycling 10 ml in tube 5 times
Step 3:	Condition column with 4 ml of 50:50 ACN/MeOH
Step 4:	Condition column with 4 ml of HPLC water
Step 5:	Load sample onto column
Step 6:	Rinse column with1.0 ml of 0.8% formic
Step 7:	Rinse column with 1.0 ml of 30:70 ACN/water
Step 8:	Dry column with gas for 120 seconds
Step 9:	Collect 1.0 ml fraction into next tube using 50:50 ACN/MeOH
Step 10:	Add 1.5 ml of ~1.5% acetic acid

Step 11:	Mix by cycling 3 ml in tube 5 times
Step 12:	Wash syringe with 10 ml of 50:50 ACN/MeOH
Step 13:	Wash syringe with 10 ml of 0.8% formic
Step 14:	END

Note the indicated benchmate parameters are guidelines and should be optimized for the instrument used. Instrument parameters may be adjusted to improve sample analysis.
It has been found useful to run at least one blank solution as a "wake up" before the actual sample runs

B. Performance Criteria

Run a set of ten 0.8% formic acid sample, surrogate fortified, blanks through the method. Set the benchmate to deliver enough internal standard per sample so that the final internal standard concentration is approximately equal to the calibration standards. Determine the standard deviation for each of the $^{13}C_6$ analytes peak area or height. If the relative standard deviation is greater than 20% for any of the analytes, optimize the instrument.

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

A. Conditions

Instrument used: Perkin Elmer Sciex API 3000 LC/MS/MS System
PE Sciex Turbo IonSpray 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: Electrospay (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 ~8.5L/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)
13C6 RPA203328:	273/165	(275)
Period 2:		
RPA202248 and IFT:	358/79	(375)
$^{13}C_6$ RPA202248 and $^{13}C_6$ IFT:	364/79	(275)

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

Note: Other brands tested have not retained RPA 202248.
Note: The column needs to be reconditioned after about 12 hours of use or whenever the RPA202248 peak has shifted to a retention time greater than about 6 minutes. To recondition, the column should be flushed with 100% acetonitrile for 15 minutes and then stored in that solvent for about 8 hours before re-use. Storing columns in mobile phase will result in extremely long retention time and a tailing peak for RPA202248. After the columns are stored they will have to conditioned with the mobile phase again for approximately 30 minutes or until the RPA202248 peak is fully separated from the RPA201772 peak.

Mobile phase flow rate: 0.400 mL/min no split

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

Injection volume: 95 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.

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.

VII. CALCULATIONS

Linear regression should be used to generate calibration curves for RPA 201772, RPA 202248, and RPA 203328. 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 or bracket samples to compensate for any minor change in instrument response. Samples should be diluted such that any peak areas or heights of the internal standards are approximately equal (±60%) to the internal standard peak responses in the calibration standards..

Linear regression coefficients should be calculated for the ratio of analyte to internal standard area or height plotted versus the ratio of analyte to internal standard concentration. 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:

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

where: Y= ratio of analyte response (area or height) to internal standard response (area or height)

A = intercept from linear regression analysis

B = slope from linear regression analysis (area ratio per concentration ratio)

X = ratio of analyte concentration in standards to internal standard concentration.

D = weight of internal standard (ng) added to sample divided by sample weight (g).

E = concentration of analyte in sample in parts per billion (ppb or ng/g)

B : Slope from linear regression

VIII. 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.