Method of Determination for Isoxaflutole (RPA 201772), and its Metabolites RPA 202248, RPA 203328, and RPA205834 in/on Agricultural Soil

I. INTRODUCTION AND SUMMARY

A. Scope

The objective of this method of determination is to establish the presence and concentration of isoxaflutole (RPA 201772), and its metabolites, RPA 202248, RPA 203328, and RPA 205834 in various agricultural soil regimes. Where applicable, the substrates are held in frozen storage, then prepared prior to analysis.

II MATERIALS AND METHODS

A.1 Equipment and Supplies

Nalgene® Wide Mouth High Density Polyethylene bottles (250 mL). Part No. 2104-0008.

Coors Büchner Filtering Funnels (OD 114 mm ID (Perf. Diameter) 95 mm). Part No. 60244 or equivalent.

Kimax Filtering Flasks (500 - mL Capacity) with stopper number 7. Part No. 27060-500 or equivalent.

Whatman Glass Microfibre Filters (9.0 cm). Part No. 1822 090 or equivalent.

Kimax Graduated Mixing Cylinders Single Metric (500 mL). Part No. 20039P-500 or equivalent.

Kimax Boiling Flasks, Short Neck, Flat Bottom with 24/40 Ground Glass Joint (125 mL, 250 mL, and 500 mL). Part No. 25055-125, -250, -500 or equivalent.

Büchi/Brinkmann Rotavapor® Evaporator Model RE-111 or equivalent.

Welch Duo Seal Vacuum Pump, Model No. 1400 or equivalent.

Reciprocating shaker, Atlab Shaker, Thomas Scientific or equivalent.

Centrifuge Marathon 10K (Capable of holding 250 - mL bottles) or equivalent.

Vac Elut SPS 24 Vacuum Manifold Varian Model 1223-4004 and repair kit or equivalent.

Liquid Chromatograph equipped with a UV or PDA detector.

Ultrasonic bath Büchi model 461 or equivalent.

Equivalents for the following may be substituted

A.2 Glassware

Assorted class "A" pipettes
Assorted class "A "graduated cylinders
Assorted class"A" volumetric flasks
Glass funnels, short stem
Disposable pipettes, Kimax nine inch
Zymark Turbo-Vap® tubes (200 mL)
Kontes rotary evaporator traps (24/40)
100 or 125, 250, and 500 mL boiling flasks
HPLC autosampler vials
24/40 glass stoppers
Assorted repeator pipettors with 24/40 joint Erlenmeyers
Repeator pipettor, 10 mL
Repeator pipettor, 20 mL
Repeator pipettor, 40 mL
Repeator pipettor, 50 mL
Repeator pipettor, 75 mL
Bottles and/or solvent bottles with caps for solutions
SPE cartridge adaptors (URG), part# URG-2440-SPECA

A.3 Apparatus

Utility cart (optional)
Zymark Turbo-Vap® (200 mL tubes) (optional)
magnetic stirrer (optional)

A.4 Miscellaneous Laboratory Supplies

Equivalents for the following may be substituted

Teflon 24/40 glassware sleeves
Teflon tape, 1/2 inch
Plastic weighting boats
Assorted cork rings
Manifold vacuum tubing
Teflon solvent dispensers
Pipette bulbs
Glass or teflon stopcocks, various sizes
Teflon stirring bars, assorted sizes
Magnetic wand for stirring bars
HPLC autosampler vial trays
Neoprene adaptors for Büchner funnels to flasks
Micro dishwashing detergent
Sparkleen dishwashing detergent
Alcotabs (optional)
Tygon tubing, 1/4, 3/8, 1/2" ID
Rubber tubing, according to vacuum apparatus
Plastic tank for dishwashing (optional)
Dispensers for solvents, wash solution, etc.
Laboratory gloves, various sizes
Absorbent paper (for bench)
Assorted label tape, various colors, ca. 1/2 inch
Interlarm interval timer
Octagonal stirring bars (3")
Teflon FEP wash bottle
Varian luer-lock stopcocks
Varian SPE adaptor
Varian SPE 60/75 cc reservoir

B. Reagents and Standards

B.1 Reagents

Water, HPLC grade

Acetonitrile, Suitable for Pesticide Residue Analysis, Burdick and Jackson Cat. No. 015-4 DK or equivalent. (Note 1)

Acetone, ChromPure™, Burdick and Jackson Cat. No. CP80100-4 DK or equivalent.

Hexane UV, Burdick and Jackson Cat. No. 216-4 DK or equivalent.

Methanol ChromPure™, Burdick and Jackson Cat. No. CP80150-4 DK or equivalent.

Trifluoroacetic acid, Spectroscopic Grade , Aldrich Cat. No 30,203-1 or equivalent.

Phosphoric acid, 99.999%, Aldrich Cat. No. 34524-5 or equivalent.

Varian C8-Solid Phase Extraction cartridge 5 gram/20 cc Part No. 1225-6024 No substitution.

Waters Diol-Solid Phase Extraction cartridge 5 gram/20 cc Part No. 54690 No substitution.

Supelco graphitized carbon, Part No. 5-7210

B.1 Reagents

Trifluoroacetic Acid

Trifluoroacetic Acid (TFA) 0.1 %: Solution for extraction. Pipet 1 mL of the concentrated TFA solution into a 1 liter volumetric measuring vessel and dilute to the mark with purified water.

Trifluoroacetic Acid (TFA) 0.4 %: Solution for extraction of California soil. Pipet 4 mL of the concentrated TFA solution into a 1 liter volumetric measuring vessel and dilute to the mark with purified water.

Trifluoroacetic Acid:Acetonitrile

20% solution of 0.1% TFA in Acetonitrile: Solution for extraction. Add 200 mL of 0.1 % TFA solution into a 1 liter volumetric measuring vessel and fill to the mark with acetonitrile.

20% solution of 0.4% TFA in Acetonitrile: Solution for extraction of California soil. Add 200 mL of 0.4 % TFA solution into a 1 liter volumetric measuring vessel and fill to the mark with acetonitrile.

Acidulated Water (Phosphoric)

This is an example of how the water could be prepared.

Approximately four liters of water is transferred into a pre-rinsed amber solvent bottle. The bottle is placed on top of a hot plate/magnetic stirrer in a fume hood and a teflon coated magnet is dropped in with a magnetic wand. The magnetic stirring plate is turned on and a rapid stirring rate is developed prior to the addition of phosphoric acid. After the initial addition of phosphoric acid (ca. 4 mL), let the solution mix for ca. 10 minutes and determine the pH with a calibrated meter. Adjust the pH to the target value (2.1 - 2.4) and allow the water to mix for ca. twenty minutes prior to the final pH evaluation. Remove the teflon stirring bar and cap the solution.

C8 cartridge:

Water:Acetonitrile for residue analysis (90:10) (v/v)
Water:Acetonitrile for residue analysis (50:50) (v/v)

Diol cartridge:

Acetone:Hexane (50:50) (v/v)

B.2 Standards

Reference Standards:

RPA 201772: analytical purity 99.7 % (Batch JYG 708)
RPA 202248: analytical purity 99.3 % (Batch JYG 803A)
RPA 205834: analytical purity 97.4 % (Batch IGB 802)
RPA 203328: analytical purity 99.8 % (Batch DAS 336)

Origin: Rhône-Poulenc Sector Agro
Centre de Recherche de la Dargoire
LYON-FRANCE

NOTE: analytical purity and batch references change over time.

Preparation of Standard Solutions:

This is an example of how it could be done.

Weigh approximately 50 mg of each compound (corrected for purity) into a 50-mL volumetric flask, dissolve in acetonitrile, and dilute to volume. This solution now contains 1 mg of each compound per mL . NOTE 1, 2

Take 10 mL of each stock solution and transfer to a 100-mL volumetric flask and dilute to the mark with water (H_3PO_4):acetonitrile (80:20) v/v to give a solution containing 0.1 mg/mL of each compound. Other dilutions may be made as required. These solutions can be used for the preparation of Spiking and LC standards.

B.2 Standards

Solutions for calibration:

Each calibration solution is prepared in water (H_3PO_4):acetonitrile (80:20) v/v.

Spiking Solutions:

These solutions are prepared by dilutions of the standard solutions in water (H_3PO_4):acetonitrile (80:20) v/v.

Reference LC Spike Solution Preparation:

A reference LC spike solution will be prepared at each recovery fortification level of the UTC sample. For a given fortification level, pipet an equal volume of the fortification mixture into an appropriately sized volumetric flask. Dilute this spike to volume and/or take an aliquot and dilute it to equal an analyte concentration similar to the soil extract aliquot taken for analysis. The final solution of the LC spike solution is prepared in water (H_3PO_4):acetonitrile (80:20) v/v.

Conservation of the standard solutions:

Solutions will be stored in a refrigerator at ca. 1°C - 10 °C at all times when not in use. Solutions should be allowed to warm to room temperature prior to use.

C Procedure

1. Weigh a ca. 50 gram aliquot of soil into a 250 mL Nalgene® bottle and perform sample fortification at this point if appropriate. (Shake the fortified soil well to induce appropriate mixing.) NOTE 3

2. Add ca. 1.0 gram of graphitized carbon to each soil sample and shake well to create a homogeneous mixture. NOTE 4

3. Add ca. 150 mL of the 0.1% TFA:Acetonitrile (20:80) v/v and shake on flat bed shaker for ca. 15 minutes (California 0.4% TFA:CH_3CH). NOTE 5, 6

C Procedure

4. Centrifuge at approximately 2000 rpm for ca. 5 minutes.

5. Decant liquid through a GF/C filter paper in a büchner funnel attached to a 500 or 1000 mL filtering flask (or directly to a 500 mL graduated cylinder capable of withstanding vacuum). Rinse the filter paper with ca. 20 mL of acetonitrile.

6. Repeat steps 3 through 5. Pour the extract into a labeled 500 mL mixing (graduated) cylinder (if it is not already attached to Büchner) and return the funnel to the flask at your conveinience.

7. Add an additional ca. 100 mL of the 0.1% TFA:Acetonitrile (20:80) v/v and shake for ca. 15 minutes (California 0.4% TFA:CH_3CH).

8. Pour the entire contents of the Nalgene® bottle into the büchner funnel and rinse the bottle, then the filter cake with two ca. 25 mL aliquots of acetonitrile.

9. Filter the soil to dryness and transfer the remaining extract into the appropriate mixing (graduated) cylinder (if it is not already attached to Büchner) and dilute to 500 mL with acetonitrile. Remove filtration assembly, cap graduated cylinder, and mix by inverting several times.

10. Take a 200-mL aliquot of the extract (EXTRACT A) and rotary evaporate (ca. 40°C) to approximately 5 mL. NOTE 7, 8

11. Sonicate the concentrated EXTRACT A well for ca. 5 min., ie., by rotating the flask for 1 minute and letting the flask remain in the bath for the remainder of the time.

12. Condition the C8 cartridge with ca. 20 mL of 50:50 water:acetonitrile v/v and then with ca. 20 mL of 90:10 water:acetonitrile v/v, do not let any surface area of column go to dryness. Discard the solvent. NOTE 9

13. Add the sample to the cartridge STOP close luer-lock stopcock. Add ca. 20 mL of 90:10 water:acetonitrile v/v to the original sample flask and sonicate well for ca. 5 minutes, then set aside until ready for solvent addition.

C Procedure

14. Open the stopcock and drain the sample using gravity to establish a flow rate of no greater than 2 mL/min. Add the sonicated rinse from step #13 to the C8 cartridge immediately following the sample elution to the top surface (frit) of the C8 cartridge. Elute the 20 mL of 90:10 water:acetonitrile v/v to the top surface (frit) of the C8 cartridge STOP close luer-lock stopcock and do not let column go to dryness. Discard the solvent when appropriate. NOTE 10

15. Replace the collection vessel with a 100 or 125 mL boiling flask, add ca. 40 mL of 90:10 water:acetonitrile v/v to the original sample flask and sonicate well, Add the sonicated 40 mL of 90:10 water:acetonitrile v/v to the cartridge, open the luer-lock stopcock and elute the 40 mL of 90:10 water:acetonitrile v/v to the top surface (frit) of the C8 cartridge STOP close the luer-lock stopcock. This fraction represents Fraction 1 which contains RPA 203328. SAVE

16. Replace the collection vessel with a 250 mL flask, add ca. 80 mL of 50:50 water:acetonitrile v/v to the original sample flask and sonicate well for ca. 5 minutes, add to the cartridge and elute the 80 mL of 50:50 water:acetonitrile v/v to the top surface (frit) of the C8 cartridge STOP close the luer-lock. This fraction represents EXTRACT B , it contains the other three compounds, RPA 202248, RPA 205834, and RPA 201772. SAVE This is a stopping point for these compounds, samples should be placed in a refrigerator or freezer if you stop here. NOTE 11

17. Concentrate Fraction 1 just to dryness on the rotary evaporator (ca. 40°C).

18. Pipet 5.0 mL of 80:20 water (H_3PO_4):acetonitrile v/v into the boiling flask containing Fraction 1 and sonicate well as before. This fraction is ready to be chromatographed.

19. Remove the samples (EXTRACT B) from the refrigerator/freezer (if necessary) and concentrate just to dryness (ca. 40°C) using a rotary evaporator. Pipet 5 mL of 50:50 acetone:hexane v/v into the flask and sonicate well as before. This is EXTRACT B.

C Procedure

20. Condition the 5 gram diol cartridge by adding ca. 20 mL of methanol followed by ca. 20 mL of 50:50 acetone:hexane v/v. Do not let the column go to dryness. Discard the solvent at this point if necessary.

21. Add concentrated EXTRACT B to the diol cartridge with the low flow rate as before, elute the 5 mL of 50:50 acetone:hexane v/v to the top surface (frit) of the diol cartridge STOP close the luer-lock stopcock and discard the solvent. Replace the collection vessel with a 100 or 125 mL boiling flask.

22. Add ca. 40 mL of 50:50 acetone:hexane v/v to the sample flask (same flask as step #19) and sonicate well as before. Load this fraction onto the cartridge, elute to the top surface (frit) of the diol cartridge and collect as Fraction 2. This fraction contains RPA 201772 and RPA 203834.

23. Replace the collection vessel with another 100 or 125 mL boiling flask, add ca. 40 mL of methanol to the sample flask (same flask as step #19) and sonicate well as before. Load this fraction onto the cartridge, elute to the top surface (frit) of the diol cartridge and collect as Fraction 3. This fraction contains RPA 202248.

24. Fractions 2 and 3 are concentrated just to dryness using a rotary evaporator (ca. 40°C) and brought up to 5.0 mL with 80:20 water (H_3PO_4):acetonitrile v/v. Sonicate well as before. NOTE 12

25. Fractions 2 and 3 can now be transferred to the appropriate HPLC vial and chromatographed on the HPLC using the specified conditions.

D. Methods of Calculation

D.1 Calculations

Residues are quantified using a linear regression curve generated from a series of standards.

Equations of the following form are used:

Calculation as parts per billion (ppb): $(F) = \frac{C - A}{B} \times \frac{D}{E}$

A = intercept determined by linear regression (ng./mL.)
B = slope determined by linear regression (response per ng./mL.)
C = response of analyte of interest
D = final sample volume
E = weight of sample in final extraction (grams)
F = sample concentration in parts per billion (ppb)

The reference LC spike solution prepared for each analytical set will also be quantified by the same calibration curve as the analyte of interest.

Fresh Fortified Sample % Recovery: $(H) = \frac{F - F\,utc}{G} \times 100$

F utc = sample concentration in parts per billion of analyte (background) in untreated soil
G = ppb found in reference LC spike
H = fresh fortified sample % recovery

Calculation for storage stability:

Storage stability sample % recovery: $(K) = \frac{I - F\,utc}{J} \times 100$

I = parts per billion in storage sample
J = fortification level(ppb) of storage sample
K = storage stability sample % recovery

D. Methods of Calculation

D.1 Calculations

Calculation for storage stability:

Corrected spike recovery: $(L) = \frac{K}{H} \times 100$

L = corrected spike recovery

D.2 Limit of Quantitation

The limit of quantitation for each compound in this method of determination is ten nanograms per gram (10 ppb).

D.3 Linearity

The HPLC detector selected should exhibit linear response utilizing a minmum three point calibration range that represents a concentration range of no greater than ten times.

E. HPLC Parameters

E.1 HPLC Parameters for RPA 203328

Detector: UV or PDA

Wavelenght: RPA 203328 270 nm.

UV/Vis: 0.01 AUFS UV/Vis Filter: 1 sec.

Pump A: Water (pH ca. 2.1 - 2.4 with H_3PO_4)
Pump B: Acetonitrile UV

Column: Alltech Alltima C-18, 5 micron, 250 X 4.6

Flow (mL/min): 0.8 mL/min.
NOTE: A 250 X 3.2 column may be used at a 0.5 mL/min. flow rate.

Guard Column: RP-18 in a guard cartridge.

Time	Flow	%A	%B
Initial Conditions.	0.8	70	30
18.0	0.8	70	30
18.1	0.8	10	90
23.0	0.8	10	90
23.1	0.8	70	30
25.0	STOP		

Retention time for a 250 x 4.6 column:

RPA 203328 ca. 16.6 min.

NOTE: Analysts must optimze their instruments.

E.1 HPLC Parameters for RPA 202248

Detector: UV or PDA

Wavelenght: RPA 202248 300 nm.

UV/Vis: 0.01 AUFS UV/Vis Filter: 1 sec.

Pump A: Water (pH ca. 2.1 - 2.4 with H_3PO_4)
Pump B: Acetonitrile UV

Column: Alltech Alltima C-18, 5 micron, 250 X 4.6

Flow (mL/min): 0.8 mL/min.
NOTE: A 250 X 3.2 column may be used at a 0.5 mL/min. flow rate.

Guard Column: RP-18 in a guard cartridge.

Time	Flow	%A	%B
Initial Conditions.	0.8	50	50
12.0	0.8	50	50
12.1	0.8	10	90
17.0	0.8	10	90
17.1	0.8	50	50
25.0	STOP		

Retention time for a 250 x 4.6 column:

RPA 202248 ca. 10.2 min.

NOTE: Analysts must optimize their instruments.

E.1 HPLC Parameters for RPA 201772 and 205834

Detector: UV or PDA

Wavelenght: RPA 201772, 205834 270 nm.

UV/Vis: 0.01 AUFS UV/Vis Filter: 1 sec.

Pump A: Water (pH ca. 2.1 - 2.4 with H_3PO_4)
Pump B: Acetonitrile UV

Column: Alltech Alltima C-18, 5 micron, 250 X 4.6

Flow (mL/min): 0.8 mL/min.
NOTE: A 250 X 3.2 column may be used at a 0.5 mL/min. flow rate.

Guard Column: RP-18 in a guard cartridge.

Time	Flow	%A	%B
Initial Conditions.	0.8	50	50
25.0	0.8	50	50
25.1	0.8	10	90
30.0	0.8	10	90
30.1	0.8	50	50
35.0	STOP		

Retention times for 250 x 4.6 column:

RPA 201772 ca. 19.2 min.
RPA 205834 ca. 9.3 min.

NOTE: Analysts must optimize their instruments.

Agricultural soil samples which exhibit quantitative interference may require the following parameters.

E.1 HPLC Parameters

Detector: UV or PDA optimized for sensitivity.

Wavelenght:

RPA 203328	270 nm.
RPA 201772	270 nm.
RPA 202248	300 nm.
RPA 205834	300 nm.

UV/Vis: 0.01AUFS UV/Vis Filter: 1.sec.

Pump A: Water (pH ca. 2.1 - 2.4 with H_3PO_4)
Pump B: Acetonitrile UV

Column: Alltech Alltima C-18, 5 micron, 250 X 4.6
Flow (ml/min): 1.0 mL/min.
NOTE: A 250 X 3.2 column may be used at a 0.5 mL/min. flow rate.

Guard Column: RP-18 in a guard cartridge.

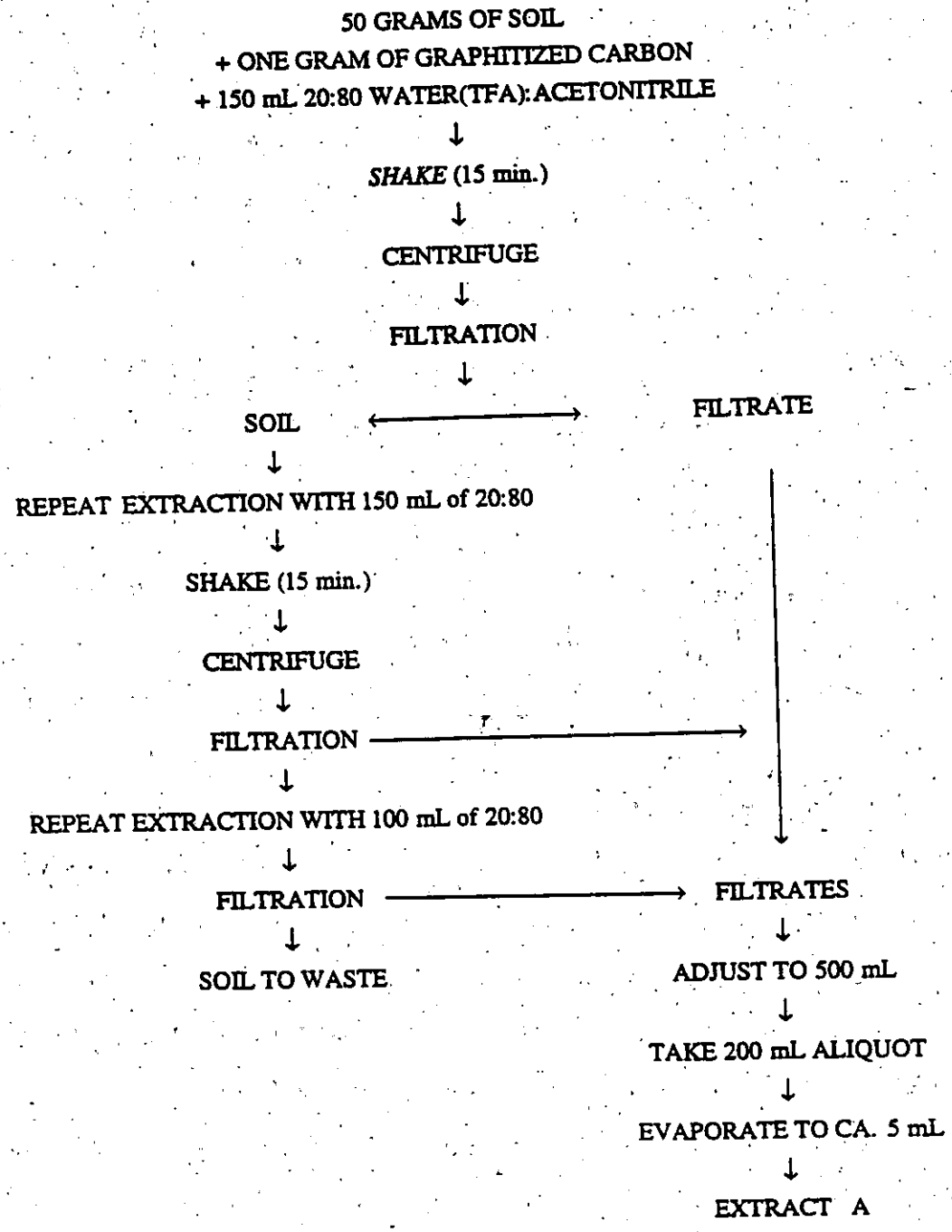
Time	Flow	%A	%B
Initial Conditions:	1.0	80	20
2.0	1.0	80	20
6.0	1.0	70	30
11.0	1.0	60	40
17.0	1.0	50	50
25.0	1.0	10	90
30.0	1.0	10	90
35.0	1.0	80	20
45.0	1.0	80	20

Retention times for 250 x 4.6 column:

RPA 203328	ca. 12.60 min.
RPA 205834	ca. 17.55 min.
RPA 202248	ca. 17.80 min.
RPA 201772	ca. 22.73 min

G.

EXTRACTION WITH SOLVENT



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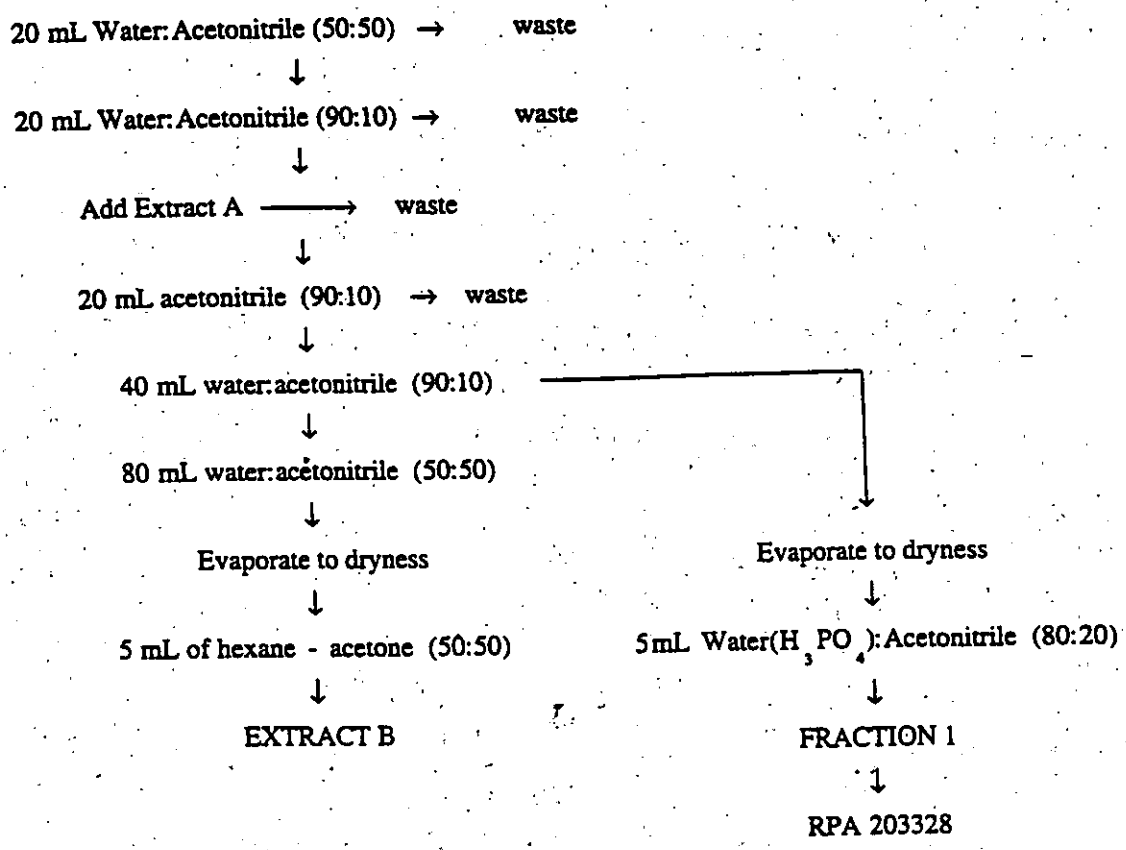
VARIAN C8 SOLID PHASE EXTRACTION



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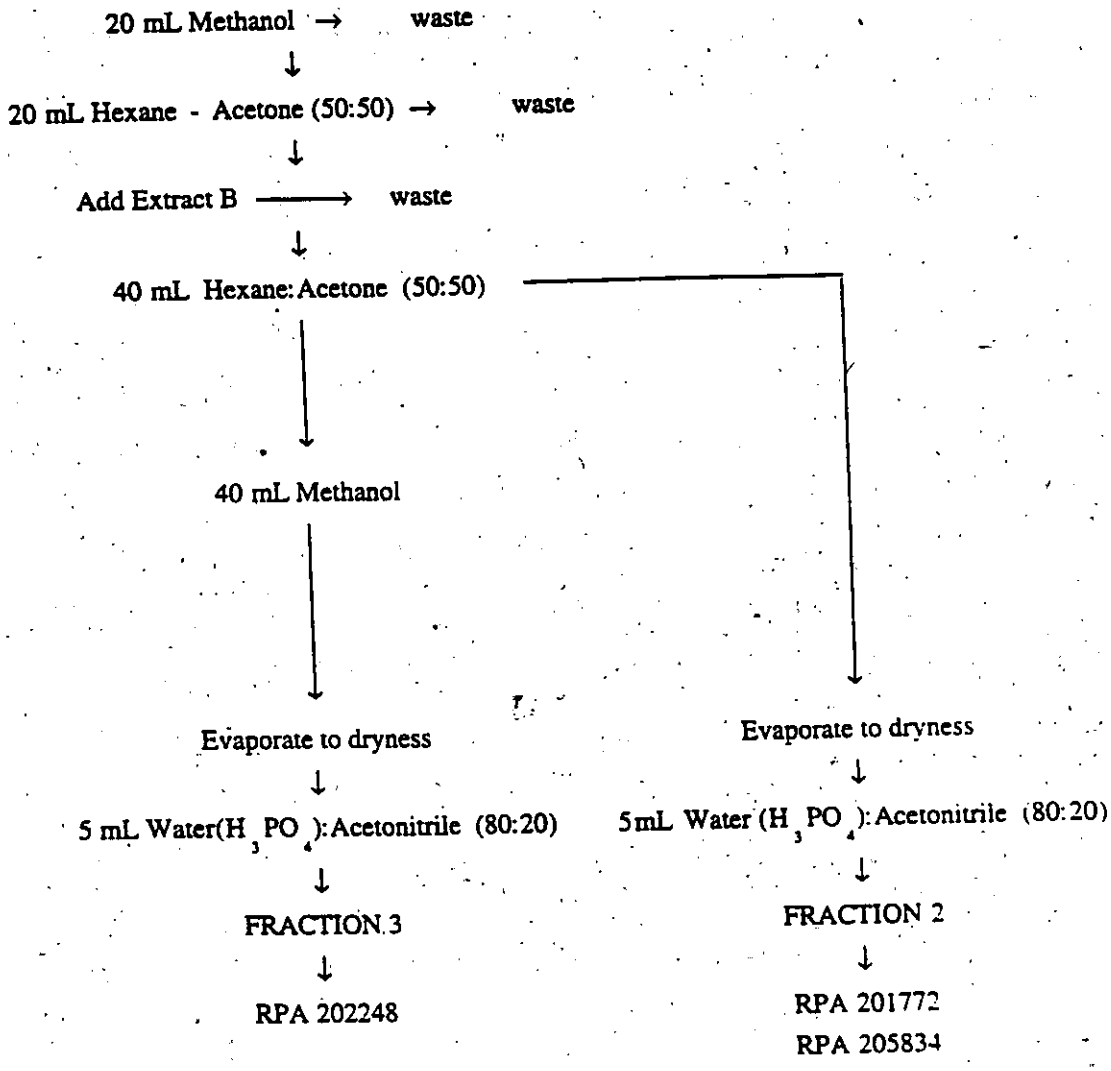
WATERS DIOL SOLID PHASE EXTRACTION



III NOTES

1. It is recommended that the wash bottles are manufactured with Teflon® FEP or any alternative which deters phthalate contamination. These wash bottles are typically used for multiple projects which may involve electron capture determination at picogram quantitation levels. Laboratory activities should be examined where plastic components utilized for this procedure may cross contaminate other projects.

2. It is recommended that bottles used for standard and fortification solutions receive an acetonitrile rinse followed by an over-nite soak in a solution of 80:20 water (H_3PO_4):acetonitrile prior to use.

3. Nalgene bottles should be rinsed with acetonitrile prior to use. It is recommended that laboratories not familiar with the methodology perform a reagent blank prior to sample analysis.

4. Ca. means approximately.

5. Add ca. one-two revolutions of teflon tape to the threads of each bottle (optional). Seal the cap tightly.

6. The type of acetonitrile used during the analysis is important. Use an acetonitrile that is suitable for pesticide residue analysis when performing the extraction or any purification steps. However, it is acceptable to use an acetonitrile UV suitable for HPLC analysis when preparing the standard solutions and for the mobile phase.

7. The rotary evaporation steps should be performed at a moderately fast easy pace so as not to incur bumping or sample carry over. Also it may be necessary to use additional acetonitrile to form an azeotrope with the larger percentage water fractions (i.e. fraction 1).

8. Another 200 mL aliquot of the sample extract may be transferred to a clean and labeled 250 mL Nalgene® bottle. The aliquot is kept in refrigerated or freezer storage until it is used or discarded.

9. It is very important not to let the SPE cartridges (C8 and Diol) go dry at any point in the procedure. Once the conditioning solvents are applied to the cartridges, the solvents can not be allowed to go below the frit on top of the packing material in the cartridge at any step. The use of Varian adaptors to suspend a reservoir above the column as well as Varian luer-lock stopcocks attached to the SPE column between its' base and that of the vacuum source are recommended.

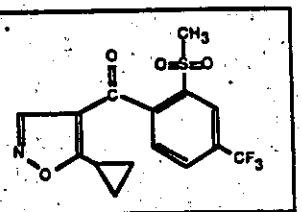
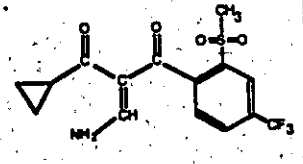
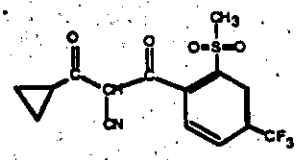
10. The rate at which the cartridges are run is also very important. It is not recommended that the rate exceed 2 mL/min. During the loading procedure of the extracts A and B, the rate should be closer to 1 mL/min. The elution solvents can run at about 2 mL/min.

11. This procedure should take two days to perform if the analytical sample set exceeds three samples, thus an appropriate stopping point is after the samples have been eluted off of the C8 cartridge. The 40 mL of 50:50 water:acetonitrile v/v should be capped and stored in a refrigerator. Fraction 1 can be concentrated to dryness and the chromatography performed if the analyst so choices.

12. A Zymark Turbo-Vap® which utilizes 200 mL Turbo-Vap® tubes may be substituted for rotary evaporation for preparation of fraction number 2 and 3 generated during diol solid phase extraction. Reduce the sample volume to ca. the tube meniscus at 40°C and 0.9 bar. Add ca. 25 mL of acetonitrile, swirl to mix, and continue reduction to ca. just below the tube meniscus. Pipette 4 mL of acidic water (H3PO4) into the Turbo-Vap® tube and swirl vigourously to mix, use a Kimax disposable pipette to rinse the lower ca. interior glass surface repeatedly prior to transfer to a 5 mL volumetric. After the Turbo-Vap® tube has been emptied, it may be necessary to acquire an aliquot of acetonitrile in the pipette for additional rinsing of the Turbo-Vap® tube and for addition to the 5 mL volumetric.

IV. FIGURES

Chemical Structures of Isoxaflutole (RPA 201772), and the Metabolites RPA 202248, RPA 203328, and RPA 205834

RPA201772	isoxazole (Parent AI)	4-(2-methanesulfonyl-4-trifluoromethylbenzoyl)-5-cyclopropyl isoxazole	
RPA205834	isoxazole "enamine"	2-aminomethylene-1-cyclopropyl-3-(2-methylsulphonyl-4-trifluoromethyl-phenyl) propan-1,3-dione	
RPA202248	isoxazole-'DKN'	2-cyano-3-cyclopropyl-1-(2-methylsulfonyl-4-trifluoromethylphenyl) propane-1,3-dione	
RPA203328	isoxazole "benzoate"	2methylsulfonyl-4-trifluoromethylbenzoic acid