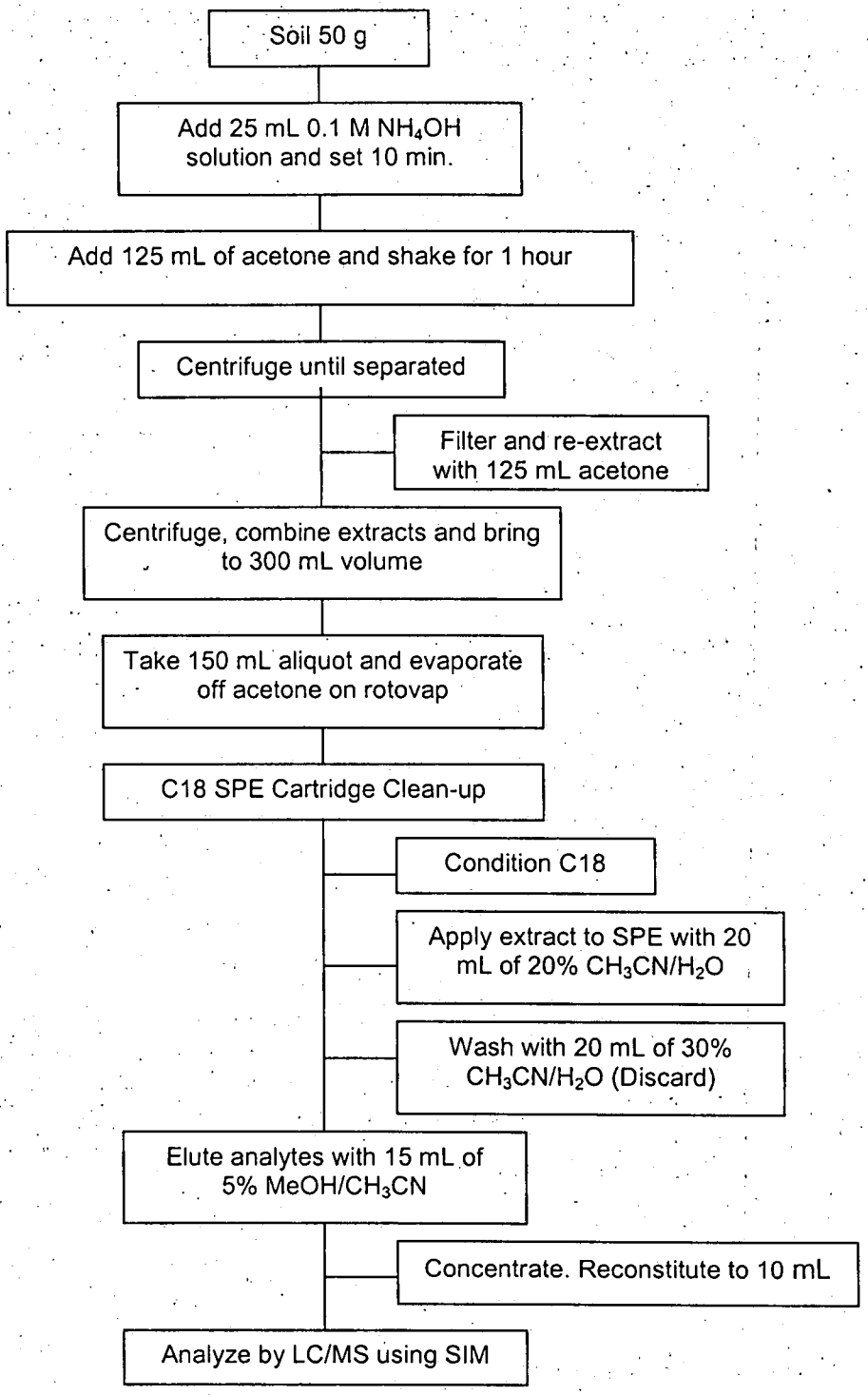
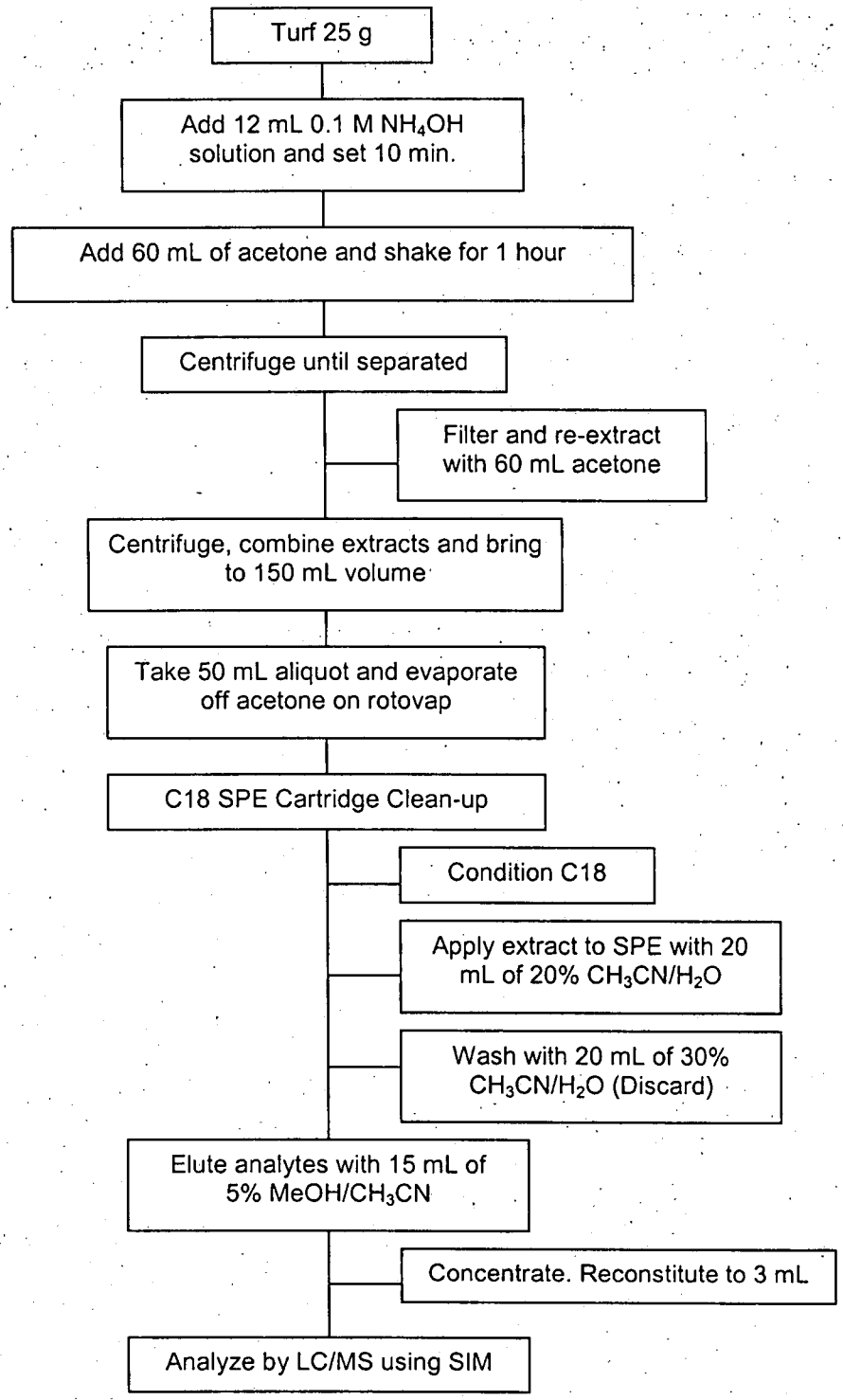
## SUMMARY FLOWCHART OF ANALYTICAL METHOD (SOIL)



## SUMMARY FLOWCHART OF ANALYTICAL METHOD (TURF)



# 1.0 INTRODUCTION

## 1.1 Scope

This method sets forth the procedure for determining the residues of RPA 400727 (Triticonazole) and its metabolites RPA 406203 and RPA 406341 in soil and turf. The method is based on Rhône Poulenc method no. AR91-82 entitled "RPA 400727 Methode de Dosage Des Residus Dans Le Sol" by M. Guillet/B. Simonin. The method was modified at Enviro-Test Labs to utilize LC/MS rather than GC/ECD.

## 1.2 Principle

An analytical method is described for the determination of residues of RPA 400727 (Triticonazole) and its metabolites RPA 406203 and RPA 406341 in soil and turf. The inclusion of metabolite RPA 407922 in the method is for demonstrating HPLC resolution of this metabolite from RPA 406341 only. Residues of RPA 400727, RPA 406203 and RPA 406341 are extracted from soil/turf using sonication and shaking with acetone/water. All residue analysis is accomplished by LC/MS (thermospray and/or ionspray) on a $C_{18}$ column. Quantitation of results is based on a comparison of peak areas with those of known standards. The method has been validated at 5 ppb, 25 ppb, and 50 ppb for RPA 400727, RPA 406203 and RPA 406341 by preparing and analyzing control and fortified soils and turf from California, North Carolina and Washington. The method has also been verified on Canadian soils at levels from 5 - 100 ppb for RPA 400727 and metabolite RPA 406341.

## 1.3 Method Limits

The method detection level (MDL) and limit of quantitation (LOQ) for RPA 400727, RPA 406203 and RPA 406341 in soil and turf were determined during validation of the method (ETL Report 98RP26.REP). The results are shown in Section 11.0, Table 1 for soil and Table 2 for turf.

The target LOQ of 0.005 ppm for all analytes in soil and turf were close to the calculated LOQ values and consequently 0.005 ppm was established as the LOQ fortification level.

## 2.0 MATERIALS

### 2.1 Reagents/Solvents

(Equivalent or better grade reagents/solvents may be substituted.)

Acetone - glass distilled, EM Science, OmniSolv®
Acetonitrile ($CH_3CN$) - glass distilled, EM Science, OmniSolv®
Ammonium acetate - ACS grade, Fisher
Glacial Acetic acid - ACS grade, Fisher
Methanol - glass distilled, EM Science, OmniSolv®
Sodium sulfate - purified by heating to 400°C
Water, deionized - Millipore Purification System

### 2.2 Equipment and Supplies

(Equivalent equipment may be substituted.)

Balance - Sartorius 1206 MP, VWR Scientific
Bottles, centrifuge - polypropylene, 250 mL, Baxter
Cartridge, C18 SPE - 2 g, Supelco Cat.No. 5-7117
Centrifuge - Sorvall®, RC2-B with 250 mL rotor head, DuPont Instruments
Centrifuge -HN-S with 8 position head, International Equipment Co. (for 40 mL tubes)
Column, HPLC - Symmetry®, C18 5 µm 4.6 × 250 mm, Waters, Cat.No. WAT05475
Culture tubes - 15 mL, screw-top tube, 16 × 25 mm, Kimble Glass Inc.
Cylinders, graduated - 100, 250 and 500 mL
Flasks, round bottom, 50 and 500 mL - Kimble Glass Inc.
Flasks, volumetric - 25 and 100 mL, Class A
Funnels, wide-mouth - polypropylene
Nitrogen evaporator with water bath - Organomation Assoc. Inc., Model No.111
Pipettes, volumetric - 10 mL
Rotoevaporator with water bath - RE51, Yamato
Rubber bulb - Fisher (to apply pressure to SPE cartridge)
Shaker, platform or wrist - Pychotherm
TurboVap tubes, 200 mL
TurboVap® nitrogen evaporator, Zymark
Ultrasonicator - Fisher Scientific, FS-28
Vacuum manifold system for SPE - Visiprep DL, Cat.No. 5-7044, Supelco

## 2.3 Solutions

The following is a list of solutions used in the analyses of soil and turf.

2.3.1 $NH_4OH$ Solution (0.1M): Add 10 mL of concentrated $NH_4OH$ to 4 L of deionized water. Mix by shaking. (Used to pre-soak soil before acetone extraction.)

2.3.2 Ammonium Acetate Solution (0.5 M): Add 155 g of ammonium acetate to 4 L of deionized water. Mix by shaking. De-gas by placing 4 L bottle in sonic bath and applying vacuum for ~ 5 minutes.

2.3.3 2% Acetic acid in HPLC $H_2O$: Add 80 mL of glacial acetic acid to 4 L of deionized water and mix by shaking. De-gas by placing 4 L bottle in sonic bath and apply vacuum for at least 5 minutes.

2.3.4 2% Acetic acid in acetonitrile: Add 80 mL of glacial acetic acid to 4 L of acetonitrile and mix by shaking. De-gas as above.

2.3.5 30% ACN in water (v/v): Add 300 mL of acetonitrile to every 700 mL of deionized water. Mix.

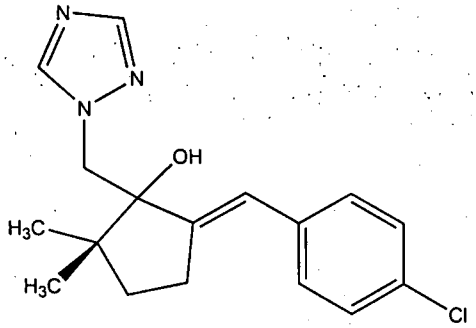
2.3.6 5% MeOH in ACN: Add 50 mL of methanol to every 950 mL of acetonitrile. Mix.

2.3.7 0.2% Acetic acid in HPLC $H_2O$: Add 8.0 mL of glacial acetic acid to 4 L of deionized water and mix by shaking. De-gas by placing 4 L bottle in sonic bath and apply vacuum for at least 5 minutes.

2.3.8 0.2% Acetic acid in acetonitrile: Add 8.0 mL of glacial acetic acid to 4 L of acetonitrile and mix by shaking. De-gas as above.

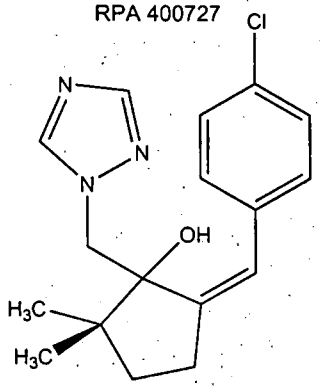
## 2.4 Analytical Standards and Chemical Structures:

RPA 400727
Chemical Name:
(1RS)-E-2-(4-chlorobenzylidene)-5,5-dimethyl-1-(1H-1,2,4-triazol-1-ylmethyl)cyclopentan-1-ol
CAS No.: 131983-72-7



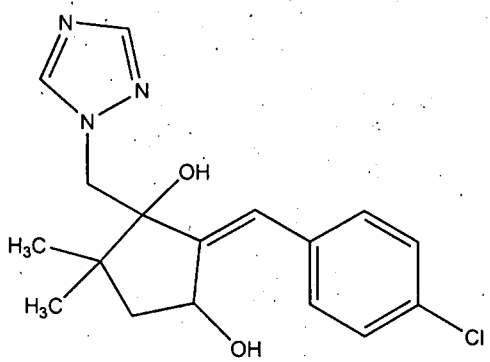
RPA 400727

RPA 406203
Chemical Name:
(1RS)-(Z)-5-(4-Chlorobenxylidene)-2,2-dimethyl-1-(1,2,4-triazol-1-ylmethyl)cyclopentan-1-ol



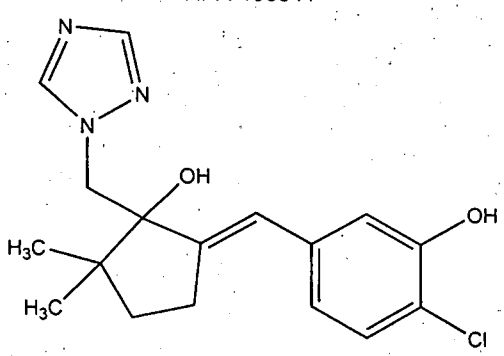
RPA 406203

RPA 406341
Chemical Name:
E-2-(4-chlorobenzylidene)-5,5-dimethyl-1-(1H-1,2,4-triazol-1-ylmethyl)-trans-cyclopentan-1,3-diol



RPA 406341

RPA 407922*
Chemical Name:
(1RS)-(E)-5-(4-chloro-3-hydroxybenzylidene)-2,2-dimethyl-1-(1,2,4-triazol-1-ylmethyl)cyclopentan-1-ol



RPA 407922

*Used in the resolution mix standard only.

## 3.0 FORTIFICATION AND CALIBRATION STANDARD SOLUTIONS

### 3.1 Preparation

All the standard solutions must be stored in glass 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 procedure for preparing a standard solution. Alternate or additional standards of appropriate weight and volume may be prepared as needed. The "~" symbol indicates approximately.

3.1.1 Accurately weigh ~ 0.025 g (corrected for purity) each of RPA 400727, RPA 406203 and RPA 406341 into separate 25 mL volumetric flasks and dilute to the mark with acetonitrile. Cap and mix by inversion. The concentration of these stock standards is ~1000 µg/mL.

3.1.2 For the preparation of fortification standards of RPA 400727, RPA 406203 and RPA 406341, transfer 10 mL of each ~1000 µg/mL standard via volumetric class "A" pipettes, to a 100 mL volumetric flask. Dilute to mark with acetonitrile. Cap and mix by inversion. The concentration of this mixed standard is ~100 µg/mL RPA 400727, RPA 406203 and RPA 406341.

3.1.3 Calibration standards for soil samples are prepared by serial dilution of the 100 µg/mL fortification standards in 20% acetonitrile/water.

Calibration standards for turf samples are prepared by serial dilution of the 100 µg/mL fortification standards in 100% acetonitrile. The peak shape of RPA 406341 is broadened for the turf samples but acetonitrile is needed in the final turf extracts to prevent precipitation of co-extractives in solution.

A standard of RPA 407922 is also prepared as in 3.1.1 and 3.1.2. A "resolution" mix standard of all 4 analytes at 10 µg/mL to 100 µg/mL is prepared and diluted to 10 and 0.100 µg/mL in 20% acetonitrile/water. It is used only for demonstrating optimization of resolution of the HPLC system.

### 3.2 Stability

3.2.1 To evaluate the stability, the following formula has been used:

$$\% \text{ Stability} = 1 - \left( \frac{\text{old standard solution}}{\text{new standard solution}} \right) \times 100$$

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.

3.2.2 Stock solutions: Each product prepared in acetonitrile and stored at 4 ± 3°C was stable for up to 18 months (ref. 99RP41A.REP).

## 4.0 METHOD PROCEDURES

### 4.1 General Notes

4.1.1 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).

4.1.2 The "~" symbol indicates approximately.

4.1.3 The elution profile of RPA 400727, RPA 406203 and RPA 406341 should be checked on each new lot of $C_{18}$ SPE cartridges. This should be done using analytes in solvent and in control matrix. A recovery of over 85% must be achieved on the SPE cartridges before proceeding. If low recoveries are observed additional fractions of elution solvent and the washes should be collected and analyzed to determine if the analytes eluted in the wash or have remained on the column.

4.1.4 The analytical C18 column must resolve the two metabolites RPA 407922 and RPA 406341 well enough to identify and quantitate RPA 406341. The gradient program may need to be modified in order to obtain this resolution. The cis/trans isomers of Triticonazole (RPA 400727 and RPA 406203) must also be resolved.

Alternate analytical columns may be substituted, provided they meet these criteria.

4.1.5 The concentration of the acetic acid in the mobile phases may be reduced to 0.2% acetic in water and 0.2% acetic in acetonitrile for the API 150 EX LC/MS, provided the LC/MS system performance is optimized.

4.1.6 It has also been found useful to split the post column flow about 5:1 (waste/source) so that about 200 uL/min. enters the turbo ionspray interface on the Sciex API 150 EX LC/MS. This has been found to increase sensitivity of all the analytes.

## 4.2 Soil Analysis of RPA 400727, RPA 406203 and RPA 406341

♦ 4.2.1 Weigh 50 g of a prepared[1] subsample of soil into a 250 mL polypropylene centrifuge bottle. Untreated control samples may be fortified at this point for determination of recovery.

4.2.2 Add 25 ± 5 mL of 0.1 M $NH_4OH$ solution to the soil, shake to mix and let sit for ~10 min.

4.2.3 Add 125 ± 5 mL of acetone to the sample and shake on wrist-action or platform shaker for ~1 hour.

♦ 4.2.4 Remove, shake by hand and place in a sonic bath for ~10 minutes.

4.2.5 Centrifuge the sample for at least 5 minutes, or until separated and decant the supernatant through a cotton ball placed in a wide-mouth funnel. Collect in a 500 mL graduated cylinder.

4.2.6 Add 125 ± 5 mL of acetone to the soil pellet in the centrifuge bottle and shake vigorously by hand.

4.2.7 Centrifuge as in 4.2.5 and combine supernatant through the sample funnel in the 500 mL graduated cylinder.

4.2.8 Adjust the volume in the cylinder to 300 mL with acetone and mix by pouring into a 500 mL boiling flask and back into the cylinder.

♦ 4.2.9 Transfer a 150 ± 2 mL aliquot (representing 25 g of soil) to the same 500 mL flask and evaporate the acetone (until only 10-15 mL of aqueous remains) using a rotoevaporator with the water bath set at 40 ±2°C. Transfer to a 40 mL screw-cap tube. Rinse 500 mL flask 2 times with 2 mL of acetonitrile and add this rinse to the 40 mL screw-cap tube. Bring to 20 mL with deionized water. Cap and centrifuge for a couple of minutes at ~1500 RPM.

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[1]Prepared subsamples are samples which have been mixed and chopped with dry ice using a Hobart food chopper. These samples must be free-flowing and homogenous prior to subsampling.

**4.2 Soil Analysis of RPA 400727, RPA 406203 and RPA 406341** cont'd

4.2.10 Set up a series of 2 g C18 SPE cartridges in a vacuum manifold and elute 15 mL of ACN followed by 15 mL of 30% $CH_3CN$/water through each cartridge. Use a rubber bulb to force the solvent through the cartridge stopping the solvent at the top of the bed (positive pressure). The conditioning rinses are discarded.

4.2.11 Transfer the 20 mL of 20% $CH_3CN$/aqueous sample extracts to the SPE cartridges leaving any solid residues in the tube. Bring to the top of the column using a combination of vacuum and positive pressure at a low rate of 1-2 drops/second and discard. Do not let the cartridge go dry! Wash cartridge with 20 mL of 30% $CH_3CN/H_2O$. Dry cartridge using vacuum (>5 mm Hg) for at least 3 minutes.

4.2.12 After a 15 mL culture tube is placed under each cartridge, elute analytes with 15 mL of 5% MeOH/acetonitrile using positive pressure.

4.2.13 The sample is concentrated on a N-Evap at 35-40°C to less than 1 mL and restored to 2 mL with acetonitrile. 8 mL of HPLC grade water is added to make a 10.0 mL final volume.

4.2.14 Transfer an aliquot to HPLC vials and analyze by LC/MS using selective ion monitoring (see section 5.0). These extracts can be stored at -20°C until analyzed.

**4.3 Turf Analysis of RPA 400727, RPA 406203 and RPA 406341**

♦ 4.3.1 Weigh 25 g of a prepared[1] subsample of turf into a 250 mL polypropylene centrifuge bottle. Untreated control samples may be fortified at this point for determination of recovery.

4.3.2 Add 12 ±2 mL of 0.1 M $NH_4OH$ solution to the turf, shake to mix and let sit for ~10 min.

4.3.3 Add 60 ±5 mL of acetone to the sample and shake on wrist-action or platform shaker for ~1 hour.

♦ 4.3.4 Remove, shake by hand and place in a sonic bath for ~10 minutes.

4.3.5 Centrifuge the sample for at least 5 minutes, or until separated and decant the supernatant through a cotton ball placed in a wide-mouth funnel. Collect in a 250 mL graduated cylinder.

4.3.6 Add 60 ± 5 mL of acetone to the pellet in the centrifuge bottle and shake vigorously by hand.

4.3.7 Centrifuge as in 4.3.5 and combine supernatant through the sample funnel in the 250 mL graduated cylinder.

4.3.8 Adjust the volume in the cylinder to 150 ± 2 mL with acetone and mix by pouring into a 250 mL or 500 mL flask and back into the cylinder.

[1]Prepared subsamples are samples which have been mixed and chopped with dry ice using a Hobart food chopper. These samples must be free-flowing and homogenous prior to subsampling.

### 4.3 Turf Analysis of RPA 400727, RPA 406203 and RPA 406341 cont'd

♦ 4.3.9 Transfer a 50 ±1 mL aliquot (representing 8.3 g of turf) to a 250 or 500 mL flask and evaporate the acetone using a rotoevaporator with the water bath set at 40 ±2°C. Transfer to a 40 mL screw-cap tube. Rinse flask 2 times with 2 mL of acetonitrile and add this rinse to the 40 mL screw-cap tube. Bring to 20 mL with deionized water. Cap and centrifuge for a couple of minutes at ~1500 RPM .

4.3.10 Set up a series of 2 g C18 SPE cartridges in a vacuum manifold and elute 15 mL of ACN followed by 15 mL of 30% $CH_3CN$/water through each cartridge. Use a rubber bulb to force the solvent through the cartridge stopping the solvent at the top of the bed (positive pressure). The conditioning rinses are discarded.

4.3.11 Transfer the 20 mL of 20% $CH_3CN$/aqueous sample extracts to the SPE cartridges leaving any solid residues in the tube. Bring to the top of the column using a combination of vacuum and positive pressure. Wash cartridge with 20 mL of 30% $CH_3CN/H_2O$. Dry cartridge using vacuum (>5 mm Hg) for at least 3 minutes.

4.3.12 After a 15 mL culture tube is placed under each cartridge, elute analytes with 15 mL of 5% MeOH/acetonitrile using positive pressure.

4.3.13 The sample is concentrated on a N-Evap to less than 1 mL at 35-40°C and restored to a 3.0 mL final volume with acetonitrile.

4.3.14 Transfer an aliquot to HPLC vials and analyze by LC/MS Turbo Ionspray using selective ion monitoring (see section 5.2). These extracts can be stored at -20°C until analyzed.

## 5.0 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY/MASS SPECTROMETRY (LC/MS)

Note: Equivalent LC/MS instrumentation may also be used and optimized to meet sensitivity requirements. LC/MS systems which utilize "heated capillary" technology may also require a "column switching" program.

### 5.1 Finnigan Thermospray LC/MS Conditions

Instruments used:

Finnigan (San Jose, CA) SSQ 710 with thermospray TSP-2 interface

Waters (Milford, MA) 600 MS systems controller

Waters 717 refrigerated autosampler

Guard Column: RP-18 Newguard 7 µ (15 × 3.2 mm), Applied Biosystems, Part # 0711-0092

HPLC column: Symmetry® C18, 5 µm, 4.6 × 250 mm
(Equivalent C18 column or guard column may be used, but metabolite resolution of RPA 406431 and RPA 407922 must be demonstrated.)

Gradient Program: (linear gradient changes)

| Time (min.) | % A | % B |
|---|---|---|
| Initial | 65 | 35 |
| 12 | 65 | 35 |
| 23 | 35 | 65 |
| 28 | 35 | 65 |
| 35 | 65 | 35 |

The above gradients may be modified to improve resolution and/or chromatography

Solvent A - (2% acetic acid/water)
Solvent B - (2% acetic acid/acetonitrile)
Flow rate - 1.2 mL/minute

Post column eluant:
Perkin Elmer HPLC/MS pump continuously adds 0.5 M aqueous ammonium acetate at a flow rate of 0.3 mL/minute.
Injection volume - 100 µL

## 5.2 PE-Sciex API-150EX LC/MS Conditions

Instruments used:

Sciex API-150EX with Turbo Ionspray Source

Varian 9012 Solvent Delivery System

Rainin AI-200 autosampler

Guard Column: RP-18 Newguard 7 µ (15.2 × 3.2 mm), Applied Biosysystems, Part # 0711-0092

HPLC column: Symmetry® C18, 5 µm, 4.6 × 250 mm
(Equivalent C18 or guard column may be used but metabolite resolution of RPA 407922 and RPA 406341 must be demonstrated.)

Gradient Program: (linear gradient changes)

| Time (min.) | % A | % B |
|---|---|---|
| Initial | 70 | 30 |
| 1.0 | 70 | 30 |
| 14 | 65 | 35 |
| 23 | 35 | 65 |
| 28 | 35 | 65 |
| 28.1 | 70 | 30 |
| 36 | 70 | 30 |

The above gradient may be modified to improve resolution and/or chromatography.

Solvent A - (0.2% acetic acid/water)
Solvent B - (0.2% acetic acid/acetonitrile)
Flow rate - 0.9 mL/minute
Injection volume - 25 µL
Split post column flow 5:1 (waste/source)

| Approximate Retention Times | | |
|---|---|---|
| | m/z Target | m/z Qualifier |
| RPA 406341 - 17.9 min. | 334.0 | 336.0 |
| RPA 407922 - 18.3 min. | 334.0 | 336.0 |
| RPA 400727 - 23.9 min. | 318.0 | 320.0 |
| RPA 406203 - 25.6 min. | 318.0 | 320.0 |

Retention times may vary from those presented above.

Example chromatograms are attached including a resolution mix standard (see Section 9.0 for API 150 EX Instrument Conditions). Note that the retention times may vary from system to system and may require optimization but peak resolution of RPA 406341 and RPA 407922 must be attained. This can normally be achieved by altering the elution gradient.

Scan type: Q1 SIM
Polarity: Positive
Acquisition mode: Profile
Pause time: 2 ms

Masses requested:

| Start Mass (amu) | Stop Mass (amu) | Step (amu) | Dwell Time (ms) |
|---|---|---|---|
| 318 | 318 | 0 | 100 |
| 320 | 320 | 0 | 100 |
| 334 | 334 | 0 | 100 |
| 336 | 336 | 0 | 100 |

ST: -16.500
RO1: -11
IQ2: 0
RO2: 0
IQ3: 0
RO3: 0
DF: -200
CEM: 2500
NEB: 15
CUR: 12
QPE: 0
POL: 0
VCM: 0
IPE: 0

Examples:

| Calibration Table Q. Calibration | |
|---|---|
| Mass | DAC value |
| 59 | 1098 |
| 175.133 | 3308 |
| 616.464 | 11711 |
| 906.673 | 17236 |
| 1254.925 | 23866 |
| 1545.134 | 29391 |
| 2010.469 | 38245 |
| 2242.637 | 42663 |

| Q1 Peak Widths | |
|---|---|
| Mass | Offset |
| 30 | 0.050 |
| 100 | 0.085 |
| 1000 | 0.483 |
| 2000 | 0.917 |

| State Table Parameters | |
|---|---|
| Parm | Value |
| IS | 5900 |
| NC | 0 |
| TEM | 475 |
| OR | 27 |
| RNG | 230 |
| QO | -2.500 |
| IQ1 | -9 |

### 5.3 Performance Criteria (LC/MS)

First Criterion:

Run a standard solution on LC/MS 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 and change instrument operating parameters.

Optimization may include altering post column split flow and/or altering the concentration of the acetic acid in the LC mobile phase.

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 0.98 for each analyte. If this criterion is met, the samples may be run with standards interspersed.

## 6.0 CALCULATIONS

Linear regression should be used to generate calibration curves for RPA 400727, RPA 406203 and RPA 406341. 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 (µg/mL) versus peak area or height. The data from the analytical standards should then be fit to the linear model.

$$y = mx + b$$

## 6.0 CALCULATIONS cont'd

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

$$\text{Conc. (ppm or ug/mL)} = \frac{(x - b)}{m} \times \frac{F.V.}{g} \times A.F.$$

NOTE: If the LC/MS system generates an intercept (b) that is >20% of the area or peak height of the LOQ spikes (0.005 ppm), then use a 1/x curve for quantitation. This will weight the low level standards and result in a curve passing nearer the origin.

Where: x = Response of analyte of interest (peak area or height)
b = Intercept from linear regression analysis (peak or height)
m = Slope from linear regression analysis (response per concentration)
F.V. = Final sample volume (mL)
g = Starting weight in grams of sample (g)

A.F. (Soil)=Aliquot factor=$\frac{\text{Extraction Solvent Volume(mL)}}{\text{Aliquot Volume (mL)}} = \frac{300\text{ mL}}{150\text{ mL}} = 2.0$

A.F. (Turf) = $\frac{150\text{ mL}}{50\text{ mL}} = 3.0$

## 7.0 SAFETY

All available appropriate MSDS's 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.

## 8.0 REVISIONS

8.1 Updated and expanded PE-Sciex API 150 EX LC/MS conditions.

8.2 Updated example chromatograms.

8.3 Added LOQ, 5 times, and 10 times LOQ method validation data tables from ETL Report 99RP48.REP.

8.4 Added additional note to Section 4.1, General Notes (0.2% acetic mobile phase).

8.5 Added Revisions, section 8.0, indicating what changes to Rev.2 were made.