1. SUMMARY

This method is suitable for the determination of the total extractable residues of AE 0172747 and its associated metabolites AE 0456148 (M6), AE 0968400 (M1), AE 1124336 (M7), AE 0941989 (M3) and AE 1392936 (M2) in soil. The lower limit of quantitation for each analyte has been set at 0.01 ppm (10ppb).

AE 0172747 and its associated metabolites were extracted from soils using microwave extraction, filtered and diluted to volume.

A deuterated internal standard containing AE 0172747-d_4, AE 0456148-d_4, AE 0968400-d_4, AE 0941989-d_4 and AE 1392936-d_5 was added to an aliquot of the extract, and analyzed for AE 0172747, AE 0456148, AE 0968400, AE 0941989 and AE 1392936 by LC/MS/MS.

An additional aliquot of the extract was analyzed by GC/MSD for AE 1124336 by external standard.

The data generated during the method validation study[1] concluded that the method detection limit (MDL) was demonstrated to fall at or below the target LOQ of 10ng/mL (ppb) for AE 0172747, AE 0456148, AE 0968400, AE 0941989, AE 1124336 and AE 1392936, with MDL's of 2, 1, 2, 2, 5 and 2ppb respectively. The calculated LOQ for AE 1124336 was determined to be 16ppb, while the calculated LOQ for AE 0172747, AE 0456148, AE 0968400, AE 0941989 and AE 1392936 were calculated to be lower than the targeted LOQ.

The mean recovery and relative standard deviation (RSD) found for AE 0172747 and its metabolites based on multiple fortifications at 10ng/mL (LOQ) and 50ng/mL (5x LOQ): were all within the range of 70 to 120% and the precision values as measured by the relative standard deviation (RSD) were all less than 20%.

2. BACKGROUND

The herbicide AE 0172747 is currently being developed by Bayer CropScience. AE 0172747 has potential uses in several crops at several different application rates. The highest anticipated rate is on corn with one pre-emergent application of 200 g ai/ha or two post emergent applications of 100 g ai/ha each. The physiochemical properties of AE 0172747 are presented in Table 1.

An analytical method was developed for the analysis of AE 0172747 and its associated metabolites AE 0456148 (M6), AE 0968400 (M1), AE 1124336 (M7), AE 0941989 (M3) and AE 1392936 (M2) in soil, and the method validated in Bayer CropScience Study Number 03RAAEX022[1]. The structures for these compounds are presented in Appendix 2. This analytical method was prepared based on the results obtained in the validation study.

Typical recovery results are presented in Appendix 3, and the data shown was obtained from both the method validation study, and from Bayer CropScience Study Number 02DO36414[2]: AE 0172747: Terrestrial Soil Dissipation Under Agricultural Field Conditions.

3. PRINCIPLE

AE 0172747 and its associated metabolites were extracted from soils using microwave extraction with 50:50 acetonitrile:0.1% acetic/water. Following extraction, the extracts were quantitatively transferred to a 250 mL graduated cylinder and diluted to volume with acetonitrile.

An aliquot of this extract was transferred to a 200 mL Zymark® Evaporation Flask, a deuterated internal standard containing AE 0172747-d_4, AE 0456148-d_4, AE 0968400-d_4, AE 0941989-d_4 and AE 1392936-d_5 was added to the extraction solution and diluted to volume with 0.1% (v/v) acetic acid in DI H_2O and analyzed by LC/MS/MS for AE 0172747, AE 0456148, AE 0968400, AE 0941989 and AE 1392936 by internal standard. (The structures of the deuterated internal standards are presented in Appendix 2.)

An additional aliquot was transferred from the original 250mL measuring cylinder to a Zymark® Evaporation Flask, evaporated to dryness and reconstituted in ethyl acetate and analyzed by GC/MSD for AE 1124336 by external standard.

A flow-chart outlining the procedure summarizes the method in Appendix 6.

Additional summaries outlining the method parameters and method characteristics are presented in Table 2 and Table 3.

4. APPARATUS

Use as a guide; equivalent apparatus may be substituted.

- VWR SuperClear Centrifuge Tubes, Polypropylene (Cat. No.: 21008-169)
- VWR Pyrex® Brand volumetric pipets, glass class A (Assorted Volumes)
- Rainin Microman® Classic positive-displacement pipettes (Cat. No.: M-50, and M-250)
- Milestone Ethos E Microwave Labstation, equipped with a Model 320 Touch Screen Controller, a PRO-16 High throughput sampler rotor and automatic temperature control with fiber optic sensor.
- Milestone 100mL Pressure Reactors.
- Porcelain Büchner funnels, 83 mm,
- Vacuum adapter, #27 stopper joint top and bottom, top female stopper joint is unground and measures 60 mm from the top of the joint to the top of the inner flair, (used for filtration directly into mixing cylinders)
- VWR Pyrex® Brand graduated cylinder (Cat. No.: 24760-100)
- VWR Pyrex® Brand volumetric flasks, glass class A (Assorted Volumes)
- VWR Pyrex® Brand disposable Pasteur pipets (Cat. No.: 53283-910 & 53283-914)
- National Scientific LC vials, Snap-Its (Cat. No.: C4011-5)
- National Scientific LC vial Snap-It Seals, (Cat. No.: C4011-55)
- Javelin Direct-Connect Column Filter, 2.1 mm, (Part No.: 88200)
- Waters SymmetryShield™ RP8 5µm 3.0 x 150 mm Column (Part No.: 186000223)
- Applied Biosystems PE Sciex 3000 LC/MS/MS System with a Dell Optiplex PC and Analyst Software Version 1.2 or higher installed.
- Applied Biosystems Turbo Ionspray Interface.
- Shimadzu LC-10AD VP HPLC pump with Gilson 215 Liquid handler and Gilson 819 Valve Actuator.
- VICI Cheminert Valve and 2 position actuator Controller.
- Zymark TurboVap II or a Büchi rotary evaporator (do not use a Zymark TurboVap LV (see Section 10.1.2))
- Zymark 200mL Evaporation Flask
- Agilent 6890 Series II GC equipped with a split/splitless injection port, a 7683 autosampler and 5973 mass selective detector. ChemStation control and processing
- GC Column- HP Ultra 1 Methyl Siloxane 12 meter x 200 µm, 0.33 µm film thickness, (HP19091A-101)
- Analytical Balance with accuracy of ± 0.01 grams for sample weights and ± 0.0001 grams for analytical standards.
- Whatman GF/A 90mm glass microfibre filter (Cat No 1820 090)
- Acrodisc ® CR13mm syringe filter with 0.45µm PTFE membrane (Cat No 4422T)

Note: Other manufacturers' equipment may be used, provided it is functionally equivalent.

5. REAGENTS

Use as a guide; equivalents or different manufactures (brands) may be substituted.

- Acetonitrile, EM Science Omnisolv, (VWR Catalog No.: EM-AX0145-1)
- Deionized Water filtered through a Milli-Q water system or:
- Water, EM Science Omnisolv, (VWR Catalog No.: EM-WX0004-1)
- Ethyl Acetate, Fisher Scientific, Optima Grade (Fischer Catalog No.: E196-4)
- Acetic Acid, Guaranteed Reagent, (VRW Catalog No.: EM-AX0073-14)
- Certified analytical reference standards of AE 0172747, AE 0456148, AE 0968400, AE 1124336, AE 0941989 and AE 1392936.
- Deuterated standards of AE 0172747-d_4, AE 0456148-d_4, AE 0968400-d_4, AE 0941989- d_4 and AE 1392936-d_5.

6. PREPARATION OF ANALYTICAL STANDARDS

NOTE: *The following procedure is an example description of how standard solutions may be prepared. Standards may be prepared as mixed solutions by dilution from individual stock solutions or prepared individually. Alternate or additional standards of appropriate weight and volume may be prepared as needed.*

Class "A" volumetric glassware or calibrated pipets should be used in the preparation of all analytical standards. All standard solutions should be stored in amber glass bottles at or below 4 ℃ when not in use. Solutions should be allowed to warm to room temperature prior to use.

6.1 Primary Stock Standard Solutions

Prepare individual 1000 µg/ml stock solutions of AE 0172747, AE 0456148, AE 0968400, AE 1124336, AE 0941989 and AE 1392936 by placing 0.0500 grams of each analyte in separate 50 mL volumetric flasks. Dilute to volume with acetonitrile.

NOTE: *Corrections for standard purities should be applied when expressing standard concentrations. For Example: If an analytical standard material has a purity of 98.0%, then 0.0510 grams (0.0500 g / 0.980) would be required to prepare a 1000 µg/ml stock solution.*

The stock standard solutions are stable for a minimum of 3 months when stored in the dark at ≤-18°C.

6.2 Fortification Standard Solutions

Prepare a stock 10 µg/mL solution containing a mixture of AE 0172747, AE 0456148, AE 0968400, AE 1124336, AE 0941989 and AE 1392936 by taking a 1 mL aliquot of each stock solution and diluting to 100 mL with acetonitrile.

Prepare a 0.1 µg/mL solution containing a mixture of AE 0172747, AE 0456148, AE 0968400, AE 1124336, AE 0941989 and AE 1392936 by taking a 1 mL aliquot of the 10 µg/mL stock solution and diluting to 100 mL with acetonitrile.

Further dilutions of this mixed fortification solution may be made as needed.

The fortification standard solutions are stable for a minimum of 3 months when stored in the dark at ≤4°C.

6.3 Deuterated Internal Standard Solutions

Prepare individual 500 µg/ml stock deuterated internal standard solutions of AE 0172747-d_4, AE 0456148-d_4, AE 0968400-d_4, AE 0941989-d_4 and AE 1392936-d_5 by placing 0.0050 grams of each analyte in separate 10 mL volumetric flasks. Dilute to volume with acetonitrile. (If a stock internal standard solution is only available diluted in solvent, adjust the weight accordingly.)

Prepare a stock 5 µg/mL solution containing a mixture of AE 0172747-d_4, AE 0456148-d_4, AE 0968400-d_4, AE 0941989-d_4 and AE 1392936-d_5 by taking a 1 mL aliquot of each stock solution and diluting to 100 mL with acetonitrile.

Prepare a stock 0.05 µg/mL solution containing a mixture of AE 0172747-d_4, AE 0456148-d_4, AE 0968400-d_4, AE 0941989-d_4 and AE 1392936-d_5 by taking a 1 mL aliquot of the 5 µg/mL AE 0172747-d_4, AE 0456148-d_4, AE 0968400-d_4, AE 0941989-d_4 and AE 1392936-d_5 solution and diluting to 100 mL with 10% acetonitrile/90% 0.1%Acetic Acid in DI water.

Further dilutions of this mixed fortification solution may be made as needed

6.4 Calibration Standard Solutions

6.4.1 LC/MS/MS Calibration Solutions

Prepare working calibration solutions consisting of 0.0, 0.05, 0.1, 0.25, 0.5, 1.0 and 2.0 ng/mL diluted to 100mL in 10:90 acetonitrile: 0.1% (v/v) acetic acid:DI H_2O. Before bringing the calibration solutions to volume, add by pipet 2.0mL of the 0.05µg/mL deuterated internal standard solution prepared in 10% acetonitrile/90% 0.1% AcOH in DI Water to each of the calibration solutions. (see Section 6.3 Deuterated Internal Standard Solutions)

Other ranges may be prepared as needed.

6.4.2 GC/MSD Calibration Solutions

Prepare working calibration solutions consisting of 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 ng/mL AE 1124336 in ethyl acetate.

Other ranges may be prepared as needed.

Both sets of calibration solutions are stable for a minimum of 3 months when stored in the dark at ≤4°C.

7. ANALYTICAL PROCEDURE FOR ANALYSIS OF SOIL

A method flow chart is presented in Appendix 6, and a summary of the analytical method parameters is presented in Table 2.

7.1 Sample Preparation

Samples should be thoroughly homogenized and stored frozen until sampled for extraction.

7.2 Extraction

Note: Fortification experiments (see Section 9.3) are not required when analysis of samples is performed for tolerance enforcement. Fortification experiments may be used for establishing & validating method efficiency as required.

7.2.1 Weigh 10 ± 0.05 grams of soil into the Milestone Ethos E Teflon pressure reactor vessel (alternatively, the sample may be weighed into a disposable centrifuge tube (50 mL) if the extraction is not being performed immediately, and transferred to the pressure reactor vessel just prior to extraction).

7.2.2 Fortify the recovery samples at the desired fortification level with the appropriate mixed standard solution prepared in acetonitrile (see Section 6.2 Fortification Stock Solutions). Let the fortified samples sit for about 10 minutes.

7.2.3 Add 50mL of 50:50 acetonitrile:0.1% acetic acid in water to each sample.

Note: If the sample was initially weighed into a disposable centrifuge tube, rinse the tube with 50mL of solvent, before adding it to the sample.

7.2.4 Add a magnetic stirrer to each reactor vessel, and insert the reactor vessel into the outer safety shield. Place the Teflon cover over the pressure reactor vessel, and

cap the outer safety shield with the 30 bar safety valve. Hand tighten the safety valve.

Note: The microwave extraction system monitors the reaction temperature using an automatic fiber optic temperature control system. The temperature sensor is directly inserted into one of the reactor vessels through a modified Teflon cover and safety valve. It is recommended that the reaction temperature is monitored using the UTC sample.

7.2.5 Load the samples into the polypropylene rotor body, and ensure that the self releasing pressure valves in each of the reactor vessels are pointing away from the rotor. Insert the fiber optic temperature control probe into the UTC pressure reactor vessel, and manually rotate the rotor body to check that the temperature control probe cable does not catch on any part of the rotor body.

7.2.6 Close the microwave door, and program the microwave with the following two methods:

Ethos E Methods	**Setting**
Extraction Method 1:	
Time (T)	5 minutes
Temperature (T1)	80°C
Watts (E)	600 max
Stirrer speed	20
Extraction Method 2:	
Time (T)	45 minutes
Temperature (T1)	130°C
Watts (E)	800 max
Stirrer speed	20

7.2.7 Extract the samples using Extraction Method 1. Once the reaction vessels have cooled to 30 to 40°C, remove the pressure reactor vessels from the microwave and release excess pressure from the reaction vessel by introducing the relief valve tool into the hole of the self-releasing valve on the pressure reactor. Ensure the external hole of the releasing valve is pointing into a fume hood.

7.2.8 Prepare a Büchner funnel lined with a Whatman GF/A filter paper into a 250 mL graduated mixing cylinder fitted with a side-arm vacuum adapter. Use acetonitrile to wet the filter paper (to ensure a good seal of the filter paper onto

the Büchner funnel), and apply a gentle vacuum. Decant the supernatant liquid through the filter assembly into the graduated cylinder.

7.2.9 Rinse the remaining sample in the reactor vessel by adding 50mL of 50:50 acetonitrile:0.1% acetic acid in water, and swirl gently for about 10 to 15 seconds. Allow the soil to settle to the bottom of the reactor vessel and decant the supernatant liquid through the Büchner funnel into the graduated cylinder.

7.2.10 Add an additional 50mL of 50:50 acetonitrile:0.1% acetic acid in water to each reactor vessel, and seal the reaction vessel using the Teflon cover and the 30 bar safety valve as described above. Ensure each sample uses the same Teflon cover and safety valve.

7.2.11 Load the samples into the microwave as described above and extract the samples using Extraction Method 2. Allow the samples to cool for to 30-40°C. Remove the pressure reactor vessels from the microwave, vent the pressure reactor and unscrew the safety cover and Teflon cap as described above.

7.2.12 Quantitatively transfer the contents of the reactor vessel into the 250mL graduated cylinder rinsing the vessel with about 50mL of acetonitrile. Rinse the filter pad with an additional 20mL of acetonitrile.

7.2.13 Remove the Büchner funnel and dilute the extract to 250mL with acetonitrile. Mix well.

Analytical Note: The procedure may be paused at this point if required. Samples should be capped and stored refrigerated until the procedure can be resumed.

7.3 AE 0172747, AE 0456148, AE 0941989, AE 0968400 and AE 1392936 analysis

7.3.1 Transfer an aliquot (typically 100mL for UTC's and LOQ samples) from 7.2.13 into a 200mL Zymark evaporation flask, volumetric flask, and add by pipet 2.0mL of the 0.05µg/mL deuterated internal standard solution prepared in 10% acetonitrile/90% 0.1% AcOH in DI Water. (see Section 6.3 Deuterated Internal Standard Solutions)

7.3.2 Evaporate to about 25 to 35mL using a Turbovap® II evaporator set to a temperature of 50°C. Dilute to 100mL with 0.1% (v/v) acetic acid:DI H_2O. Mix the sample well. Filter the sample using an Acrodisc® 0.45µm syringe filter into a LC vial and cap to await analysis by LC/MS/MS.

7.4 AE 1392936 analysis

7.4.1 Transfer an aliquot (typically 25mL) from 7.2.13 into a 200 mL Zymark evaporation flask. Evaporate to dryness using a Turbovap® II evaporator set to a temperature of 50°C. Re-dissolve the residue in 10mL of ethyl acetate. Mix the sample well. Filter the sample using an Acrodisc® 0.45µm syringe filter into a GC vial and cap to await analysis by GC/MSD.

8. ANALYTICAL PROCEDURE FOR ANALYSIS OF APPLICATION PADS

As part of soil field dissipation studies, the application rate may be verified by the analysis of filter paper dosimeter plaques. The filter paper plaques are set out on the field prior to the application of the test substance and retrieved immediately after that application.

Use the method below as a guide only, the exact method used will depend on both the size of the plaques used and the expected concentration of AE 0172747 on each of the pads.

The method described below was based on the analysis of four filter papers, each with a diameter of 9cm. The AE 0172747 application rate as 200g ai/ha, which results in a theoretical concentration of 127µg AE 0172747 on each of the plaques.

8.1 Extraction

8.1.1 Remove the four filter papers from the sealed plastic bag, and place in a mason jar. Rinse out the plastic bags with two aliquots of ~10mL 50:50 water:0.1% acetic acid, adding the rinsate to the mason jar.

8.1.2 Prepare a UTC and fortified sample. Prepare the fortified sample by spiking four similar sized filter papers with 500ppm of AE 0172747 and 10ppm of AE 0456148, AE 0968400, AE 1124336, AE 0941989 and AE 1392936.

8.1.3 Add sufficient solvent (50:50 water:0.1% acetic acid) to the mason jar to cover the filter papers (about 250mL).

8.1.4 Cap the mason jar securely and shake the jar for about 2 hours ± 10 minutes.

8.1.5 Prepare a Büchner funnel lined with a Whatman GF/A filter paper into a 500 mL graduated mixing cylinder fitted with a side-arm vacuum adapter. Use acetonitrile to wet the filter paper (to ensure a good seal of the filter paper onto the Büchner funnel), and apply a gentle vacuum. Decant the supernatant liquid through the filter assembly into the graduated cylinder.

8.1.6 Rinse the mason jar with 2 x 50mL of acetonitrile, transferring the rinsate to the 500mL measuring cylinder. Dilute the contents of the flask to 500mL with acetonitrile.

8.2 AE 0172747, AE 0456148, AE 0941989, AE 0968400 and AE 1392936 analysis

8.2.1 For AE 0172747, transfer an 100µL aliquot from 8.1.6 into a 100mL volumetric flask, add by pipet 2.0mL of the 0.05µg/mL deuterated internal standard solution prepared in 10% acetonitrile/90% 0.1% AcOH in DI Water. (see Section 6.3 Deuterated Internal Standard Solutions).

8.2.2 Dilute to 100mL with 0.1% (v/v) acetic acid:DI H_2O. Mix the sample well. Filter the sample using an Acrodisc® 0.45µm syringe filter into a LC vial and cap to await analysis by LC/MS/MS.

8.2.3 For AE 0456148, AE 0941989, AE 0968400 and AE 1392936 analysis repeat steps 8.2.1 and 8.2.2, but transfer a 5mL aliquot from 8.1.6.

8.3 AE 1392936 analysis

8.3.1 Transfer a 1mL aliquot from the 500mL measuring cylinder into a 200 mL Zymark evaporation flask. Evaporate to dryness using either a Turbovap® II evaporator set to a temperature of 50°C or using a rotary evaporator. Re-dissolve the residue in 10mL of ethyl acetate. Mix the sample well. Filter the sample using an Acrodisc® 0.45µm syringe filter into a GC vial to await analysis by GC/MSD.

9. ANALYSIS

9.1 Sample Analysis

AE 0172747, AE 0456148, AE 0941989, AE 0968400 and AE 1392936 analysis

AE 0172747, AE 0456148, AE 0968400, AE 0941989 and AE 1392936 are analyzed by LC/MS/MS by internal standard.
Inject a 15 µl aliquot of each test sample (or fortified sample matrix) from step 7.3.2 into the LC/MS/MS under the conditions presented in Appendix I. Variations in equipment or sample characteristics may require different injection volumes or slight modifications in the chromatographic or detector conditions listed in order to obtain adequate chromatographic peak shapes or sensitivity.

Note: *If the response for any of the peaks of interest is outside the range of the calibration curve, transfer a smaller aliquot from the original measuring cylinder in Section 7.2.13 into a clean 200mL Zymark evaporation flask,*

evaporated to 25 to 35mL, and add by pipet 2.0mL of the 0.05µg/mL deuterated internal standard solution and dilute to 100mL to await analysis by LC/MS/MS.

It is often beneficial to make several 'priming' injections of standards and/or samples prior to starting the LC/MS/MS analysis. Typically 4 to 6 priming injections are made. The results from these injections are not included in any calculations used in residue determinations. These injections help stabilize the LC/MS/MS response prior to running the analytical set.

AE 1392936 analysis

AE 1124336 is analyzed by GC/MSD by external standard. Inject a 2 µl aliquot of each test sample (or fortified sample matrix) from step 7.4.1 into the GC/MSD under the conditions presented in Appendix I. Variations in equipment or sample characteristics may require different injection volumes or slight modifications in the chromatographic or detector conditions listed in order to obtain adequate chromatographic peak shapes or sensitivity. Make dilutions as necessary to maintain the response within the upper concentration range of the standard calibration curve.

It is often beneficial to make several 'priming' injections of standards and/or samples prior to starting the GC/MSD analysis. Typically 4 to 6 priming injections are made. The results from these injections are not included in any calculations used in residue determinations. These injections help stabilize the GC/MSD response prior to running the analytical set.

9.2 Standard Calibration

9.2.1 LC/MS/MS Standard Calibration and Residue Calculations

Standardize the LC/MS/MS response under the conditions outlined in Appendix 1 by injecting a 15 µL aliquot of each LC/MS/MS calibration solution interspersed with samples. Construct a standard curve for the analytes of interest by plotting the ratio of the native peak count/internal standard count vs. the standard concentration. Obtain the least square regression line of this data.

Compare the peak responses (area or height) of the analyzed sample with the standard curve. Calculate the total residue concentration (R) using *Equation 1* as follows:

$$R(ppm) = \{[(Y - b)/m]/C\} \qquad \textit{(Equation 1)}$$

Where: Y = ratio of native peak(cts.)/internal standard peak (cts.)
b = Y-intercept of standard regression line
m = slope of standard regression line
C = soil/solvent ratio (g/mL)

The soil/solvent ratio *"C"* is defined by the concentration of sample in g/mL at injection using *Equation 2.* This factor incorporates all aliquots taken and dilutions made during sample work-up.

$$C = {}^{W}/_{V_1} \times {}^{V_2}/_{V_3} \qquad \textit{(Equation 2)}$$

Where: W = 10.0 grams for sample weight
V_1 = 250 mL
V_2 = 100 mL
V_3 = 100 mL

9.2.2 GC/MSD Standard Calibration and Residue Calculations

Standardize the GC/MSD response under the conditions outlined in Appendix 1 by injecting a 2.0 µL aliquot of each GC/MSD calibration solution interspersed with samples. Construct a standard curve for the analytes of interest by plotting the response vs. the standard concentration. Obtain the least square regression line of this data.
Compare the peak responses (area or height) of the analyzed sample with the standard curve. Calculate the total residue concentration (R) using *Equation 3* as follows:

$$R(ppm) = \{[(Y-b)/m]/C\} \qquad \textit{(Equation 3)}$$

Where: Y = peak response (cts.)
b = Y-intercept of standard regression line (cts.)
m = slope of standard regression line (cts mL/µg)
C = soil/solvent ratio (g/mL)

The soil/solvent ratio *"C"* is defined by the concentration of sample in g/mL at injection using *Equation 4.* This factor incorporates all aliquots taken and dilutions made during sample work-up. Further dilutions may be required to maintain the response within the range of the standard calibration curve as indicated by the variable *D.*

$$C = {}^{W}/_{V_1} \times {}^{V_2}/_{V_3} \times D \qquad \textit{(Equation 4)}$$

Where: W = 10.0 grams for sample weight
V_1 = 250 mL
V_2 = 25 mL
V_3 = 10 mL

The dilution factor D is defined by *Equation 5* below:

$$D = V_4 / V_5 \qquad \textit{(Equation 5)}$$

Where: V_4 = Aliquot taken in mL at the final volume
V_5 = Total volume in mL of the dilution

9.3 Fortification Experiments (Optional for Tolerance Enforcement)

Note: *Fortification experiments may be performed as needed to monitor method efficiency and reproducibility, but are not required when analysis of samples is performed for tolerance enforcement. Fortification experiments are intended to be used for data collection methods or establishing & validating method efficiency.*

9.3.1 With each sample set, analyze an untreated control sample and one or more fortified control samples. Calculate recoveries by *Equation 6* as follows:

$$\text{Recovery}(\%) = \frac{R - S}{T} \times 100 \qquad \textit{(Equation 6)}$$

Where: R = ppm of target analyte found in fortified sample
S = ppm of target analyte found in control sample, real or apparent
T = theoretical ppm in fortified sample

Recoveries are determined by analyzing fortified control samples alone or in conjunction with a sample set. Samples may be fortified prior to extraction at the LOQ of 0.01ppm in soil or other appropriate level with fortification solutions. Calculate the final residue R for the control (S) and fortified control (R) samples.

Note: See Appendix 5 for typical untreated control and fortified control chromatograms.

10.1.3 Time Considerations

A set of fourteen samples can be prepared in an eight hour day, analyzed overnight and the data processed the following working day.

10.1.4 Analytical stopping points *(IF NEEDED)*

As noted in the method, the procedure may be paused if needed. These should flexibly accommodate the analyst's normal working day or schedule. It is assumed that the analysis will resume during the next working period.

10.2 Critical Steps and Observations

During the development of the microwave extraction procedure, it was observed that while high temperatures were required to extract some of the metabolites from some soil types, that AE 0172747 could degrade to AE 0941989 using these extraction conditions. A two step extraction system was therefore developed, in which AE 0172747 was initially extracted using milder conditions, the supernatant extraction solution removed, fresh solvent added and a second extraction performed using harsher extraction conditions.

Therefore it is recommended that before proceeding with any analyses, the analyst should individually fortify six soil samples with each of the six test compounds, and analyze them in accordance with this analytical method to ensure that there is no degradation of any of the test compounds.

Table 2 Analytical Method Summary Parameters

Analyte(s)	AE 0172747, AE 0456148, AE 0968400, AE 0941989 and AE 1392936	AE 1124336
Extraction solvent / Technique	Microwave extraction using solvent system of acetonitrile and 0.1% acetic acid in water	
Cleanup Strategies	Sample Dilution	
Instrument Detector Column	- Shimadzu LC-10AD VP HPLC pump with Gilson 215 Liquid handler and Gilson 819 Valve Actuator - Applied Biosystems API 3000 MS/MS - Waters SymmetryShield RP8 5µM	- Agilent 6890 GC with Agilent 7683 autosampler - Agilent 5973 MSD - HP Ultra 1 Methyl Siloxane 12 m. x 200 µm
Standardization Method	Multi point calibration curve (Internal standard)	Multi point calibration curve (External standard)
Stability of Standard Solutions	Stock standard solutions are stable for a minimum of 3 months when stored in the dark at ≤–18°C Fortification and calibration standard solutions are stable for a minimum of 3 months when stored in the dark at ≤4°C	
Retention times	AE 0172747 (10.7 min), AE 0456148 (14.2), AE 0968400 (9.3), AE 0941989 (8.2) and AE 1392936(13.1)	AE 1124336 (12.2 min)

Appendix 1 Instrument Conditions

Equipment with equivalent or better sensitivity and performance may be substituted.

LC/MS/MS Parameters

NOTE: *As the LC/MS/MS system is used over time, system components slowly and gradually become contaminated which in turn decreases system performance. The chromatographic response and/or peak shape of one or more of the analytical targets may be gradually effected over time. Therefore, the given LC/MS/MS parameters listed below are guidelines of where to start. Each instrument has its own unique personality. Variations in equipment or sample characteristics may require slight modifications in the chromatographic or detector conditions listed in order to obtain adequate chromatographic peak shapes or sensitivity. These parameters should be optimized for the instrument and column actually used. Instrument parameters and mobile phase may be adjusted to improve separation from interfering peaks.*

Acquisition Parameters

Instrument Used:	Perkin Elmer Sciex API 3000 LC/MS/MS System with Valco Divert Valve	
Interface:	PE Sciex Turbo Ion Spray Electrospray	
Synchronization Mode:	LC Sync	
AutoEquilibration:	Off	
Acquisition Duration:	19 min. 0 sec.	
Number of Scans:	754	
Periods in File:	2	
Acquisition Module:	Acquisition Method	
Software Version:	Analyst 1.2	
Period 1:	Period Delay:	0.00 sec.
	Scans In Period:	504
	Relative Start Time:	0.00 msec.
	Experiments in Period:	1
Period 1 Experiment 1: Duration 8.5 Minutes	Scan Type:	MRM
	Polarity:	Positive
	Scan Mode:	N/A
	Resolution Q1:	Unit
	Resolution Q3:	Unit
	Intensity Threshold:	0 counts
	Smart Settling:	Off
	Settling Time:	700.0000 ms
	MR Pause:	5.0070 ms
	MCA:	No
	Step Size:	0.00 amu

Appendix I LC/MS/MS Parameters (con't)

Analyte (8.1 Min.)	Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	Parameter	Start	Stop
AE 0941989	405.0	228.2	500	DP	97.0	97.0
				FP	375.0	375.0
				EP	10.0	10.0
				CE	55.00	55.00
				CXP	22.00	22.00

Analyte (8.1 Min.)	Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	Parameter	Start	Stop
AE 0941989-d_4	409.0	231.0	500	DP	97.0	97.0
				FP	375.0	375.0
				EP	10.0	10.0
				CE	55.00	55.00
				CXP	22.00	22.00

Parameter Table (Period 1 Experiment 1):	NEB:	7.0
	CUR:	11.0
	IS:	4200.0 volts
	TEM:	500. ° C
	CAD:	10.00
	AUX Gas	~8.5 mL/min

Period 2:	Period Delay:	0.00 sec.
	Scans In Period:	250
	Relative Start Time:	8.5 min.
	Experiments in Period:	1

Period 2 Experiment 1: Duration 8.5 Minutes	Scan Type:	MRM
	Polarity:	Negative
	Scan Mode:	N/A
	Resolution Q1:	Unit
	Resolution Q3:	Unit
	Intensity Threshold:	0 counts
	Smart Settling:	Off
	Settling Time:	700.0000 ms
	MR Pause:	5.0070 ms
	MCA:	No
	Step Size:	0.00 amu

Appendix I LC/MS/MS Parameters (con't)

Analyte (9.2 Min.)	Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	Parameter	Start	Stop
AE 0968400	316.93	139.99	250	DP	-49.0	-49.0
				FP	-250.00	-250.00
				EP	-9.00	-9.00
				CE	-39.00	-39.00
				CXP	-6.00	-6.00
Analyte (9.2 Min.)	Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	Parameter	Start	Stop
AE 0968400-d_4	320.95	142.92	250	DP	-90.00	-90.00
				FP	-340.00	-340.00
				EP	-10.00	-10.00
				CE	-38.00	-38.00
				CXP	-7.00	-7.00
Analyte (10.7 Min.)	Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	Parameter	Start	Stop
AE 0172747	438.97	402.93	250	DP	-41.00	-41.00
				FP	-180.00	-180.00
				EP	-10.00	-10.00
				CE	-18.00	-18.00
				CXP	-11.00	-11.00
Analyte (10.7 Min.)	Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	Parameter	Start	Stop
AE 0172747-d_4	442.97	406.91	250	DP	-46.00	-46.00
				FP	-250.00	-250.00
				EP	-10.00	-10.00
				CE	-20.00	-20.00
				CXP	-11.00	-11.00
Analyte (13.9 Min.)	Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	Parameter	Start	Stop
AE 1392936	262.78	188.81	250	DP	-23.00	-23.00
				FP	-130.00	-130.00
				EP	-12.00	-12.00
				CE	-17.00	-17.00
				CXP	-11.00	-11.00
Analyte (13.9 Min.)	Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	Parameter	Start	Stop
AE 1392936-d_5	267.89	191.78	250	DP	-32.00	-32.00
				FP	-100.00	-100.00
				EP	-10.00	-10.00
				CE	-19.00	-19.00
				CXP	-15.00	-15.00
Analyte (14.8 Min.)	Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	Parameter	Start	Stop
AE 456148	344.88	300.74	250	DP	-34.00	-34.00
				FP	-160.00	-160.00
				EP	-10.00	-10.00
				CE	-12.00	-12.00
Analyte (14.8 Min.)	Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	Parameter	Start	Stop
AE 0456148-d_4	348.88	304.92	250	DP	-73.00	-73.00
				FP	-340.00	-340.00
				EP	-9.00	-9.00
				CE	-13.00	-13.00
				CXP	-6.00	-6.00

Appendix I LC/MS/MS Parameters (con't)

Parameter Table (Period 2 Experiment 1):	NEB:	7.00
	CUR:	11.00
	IS:	-4200.0 volts
	TEM:	500.° C
	CAD:	10.00
	AUX Gas	~8.5 mL/min
Autosampler Used:	Gilson 215 Autosampler	
Syringe Size:	500 µL	
Injection Volume:	15 µL	
Pre-inject Flushes:	4	
Post inject Flushes:	4	
Air Cushion:	10 µL	
Excess Volume:	10 µL	
Sample Speed:	5.00 mL/min.	
Needle Level	75%	
Inject Delay Time:	0.00 min.	
Needle Z-Direction Speed	Very Fast	
Inject Time Delay	0.0 min.	
Loop Volume:	100 µL	
Needle Flush Volume:	250 µL	
Flush Speed	5.00 mL/min.	
Port Flush Volume	400 µL	
Pump Used:	Shimadzu LC-10AVP (High Pressure Mixer)	
Minimum Pressure:	0.0 psi	
Maximum Pressure:	5000 psi	
Shutdown Time:	999.9 min.	
Guard Column:	Javelin-Direct Connect Column Filter (2.1 mm i.d.)	
Column Temperature:	Ambient	
Column:	Manufacturer:	Waters
	Type:	SymmetryShield
	Phase:	RP8
	Particle Size:	5 µM
	Diameter:	3.0 mm
	Length:	150 mm
	Pore Size:	100 Å
	Mobile Phase A:	Acetonitrile
	Mobile Phase B:	0.1% (v/v) Acetic Acid

Appendix I LC/MS/MS Parameters (con't)

Gradient Program:

Step	Total Time (min.)	Flow	Gradient	A(%)	B(%)	C(%)	D(%)	TE#1	TE#2
0	0.00	300 µL/min.	0	20.0	80.0	0.0	0.0	open	open
1	14.00	300 µL/min.	-3	99.0	1.0	0.0	0.0	open	open
2	14.99	300 µL/min.	0	99.0	1.0	0.0	0.0	open	open
3	15.00	300 µL/min.	0	20.0	80.0	0.0	0.0	open	open
4	19.00	300 µL/min.	0	20.0	80.0	0.0	0.0	open	open

Divert Valve Program:

Step	Total Time (min.)	Divert Location
1	0.0 - 5.9	To Waste
2	6.0 - 15.8	To LC/MS
3	15.9 - End	To Waste

Appendix I GC/MSD Parameters

NOTE: *As the GC/MSD system is used over time, system components slowly and gradually become contaminated which in turn decreases system performance. The chromatographic response and/or peak shape of one or more of the analytical targets may be gradually effected over time. Therefore, the given GC/MSD parameters listed below are guidelines of where to start. Each instrument has its own unique personality. Variations in equipment or sample characteristics may require slight modifications in the chromatographic or detector conditions listed in order to obtain adequate chromatographic peak shapes or sensitivity. These parameters should be optimized for the instrument and column actually used. Instrument parameters and mobile phase may be adjusted to improve separation from interfering peaks.*

Instrument Used: Agilent 6890 Series II GC

Instrument Conditions:

Oven:	Initial Temp:	90°C		
	Initial Time	2.00 min.		
	Ramp #:	Rate	Final Temp	Final Time
	1	10.00 °C/min.	215 °C	0.5 min.
	2	60.00 °C/min.	280 °C	3.0 min.
	Post Temp::	0°C		
	Post Time:	0.00 min.		
	Run Time:	19.08 min.		
	Max Temp:	325 °C		
	Equilib. Time:	0.50 min.		
Inlet:	Mode	Pulsed Splitless		
	Initial Temp:	275 °C		
	Pressure:	10.00 psi		
	Pulse Pressure:	60.0 psi		
	Pulse Time:	0.50 min.		
	Purge Flow:	60.3 mL/min.		
	Purge Time:	1.50 min.		
	Total Flow	64.8 mL/min.		
	Gas Saver:	Off		
	Gas Type:	Helium		

Appendix I GC/MSD Parameters (con't)

Column:	Hewlett Packard	Ultra 1 Methyl Siloxane (HP19091A-101)		
	Max Temp:	325 °C		
	Nominal Length:	12.0 meter		
	Nominal Diameter:	200 uM		
	Nominal Film Thickness	0.33 uM		
	Mode:	Ramped Pressure		
	Initial Time:	14.00 min.		
Column:	Ramp #:	Rate	Final Pres.	Final Time
	1	Off	----	----
	Post Pressure:	10 psi		
	Nominal Initial Flow:	1.0 mL/min.		
	Average Velocity:	59 cm/sec.		
	Inlet:	Front Inlet		
	Outlet:	MSD		
	Outlet Pressure:	Vacuum		
Detector:	Agilent 5973 Mass Selective Detector			
	Initial Temp:	280 °C		
	Initial Time:	0.00 min		
	Ramp #:	Rate	Final Temp.	Final Time
	1	Off	----	----
	Tune File:	ATUNE.U		
	Acquisition Mode:	SIM		
	Solvent Delay	10.0 min.		
	EM Absolute	False		
	EM Offset	506		
	Resulting Voltage	1389		

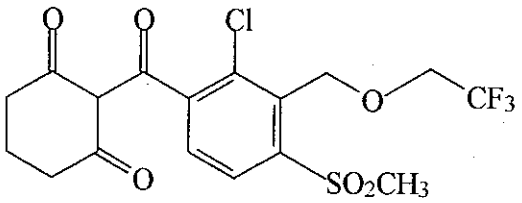
Appendix I GC/MSD Parameters (con't)

SIM Parameters:	Group 1		
	Group ID	P7	
	Resolution	Low	
	Group Start Time	0.00	
	Ions/Dwell In Group	Ion Mass	Dwell Time
		233.0	100 msec.
		249.0	100 msec.
		251.0	100 msec.
	MS Quad Temp:	150 °C	
	MS Source Temp:	230 °C	
	Timed Events	MS Off @ 15.0 min.	
Injector:	Agilent 7873 autosampler		
	Sample Washes;	3	
	Sample Pumps:	3	
	Injection Volume	2 uL	
	Syringe Size	10 uL	
	Post Inj Solvent A Washes	4	
	Post Inj Solvent B Washes	4	
	Viscosity Delay	0 sec.	
	Plunger Speed	Fast	
	Pre-Injection Dwell	0 min.	
	Post-Injection Dwell	0 min.	

Appendix 2 Structures

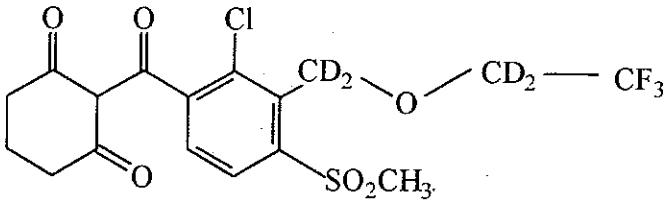
The structures for AE 0172747 and its metabolites are presented below:

Code Name: AE 0172747
(Active Ingredient, Parent Molecule)



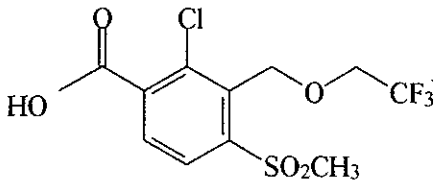
CAS Name: 2-[2-chloro-4-mesyl-3-((2,2,2-trifluroethoxy)methyl)benzoyl]cyclohexane-1,3-dione
Molecular Formula: $C_{17}H_{16}O_6ClF_3S$
Molecular Weight: 440.82
CAS Number 335104-84-2

Code Name: AE 0172747-*trifluoroethoxymethyl*-d_4
(Parent Molecule, Deuterated Internal Standard)



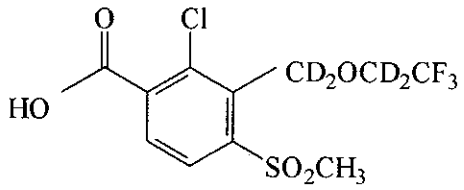
CAS Name: 2-[2-chloro-4-mesyl-3-((2,2,2-trifluroethoxy)methyl)benzoyl]cyclohexane-1,3-dione-d_4
Molecular Formula: $C_{17}H_{12}D_4O_6ClF_3S$
Molecular Weight: 444.90

Code Name: AE 0456148 (or M6)
(Soil Metabolite)



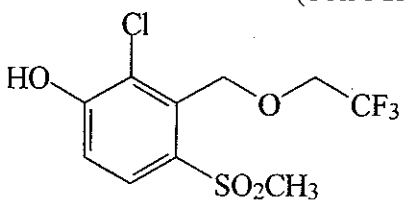
CAS Name: 2-[2-chloro-4-mesyl-3-[(2,2,2-trifluroethoxy)methyl]benzoic acid
Molecular Formula: $C_{11}H_{10}O_5ClF_3S$
Molecular Weight: 346.71

Code Name: AE 0456148-*trifluoroethoxymethyl*-d_4
(Soil Metabolite, Deuterated Internal Standard)



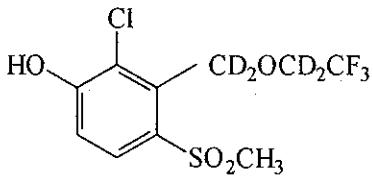
CAS Name: 2-[2-chloro-4-mesyl-3-((2,2,2-trifluroethoxy)methyl)benzoic acid-d_4
Molecular Formula: $C_{11}H_6D_4O_5ClF_3S$
Molecular Weight: 350.70

Code Name: AE 0968400 (or M1)
(Soil Metabolite)



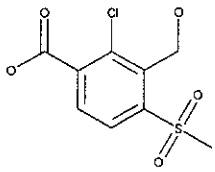
CAS Name: 2-chloro-4-(methylsulfonyl)-3-[(2,2,2-trifluoroethoxy)methyl]phenol
Molecular Formula: $C_{10}H_{10}O_4ClF_3S$
Molecular Weight: 318.70

Code Name: AE 968400-*trifluoroethoxymethyl*-d_4
(Soil Metabolite, Deuterated Internal Standard)



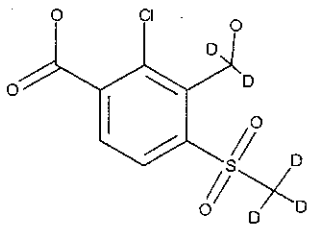
CAS Name: 2-chloro-4-(methylsulfonyl)--3-[(2,2,2-trifluoroethoxy)methyl]phenol-trifluoroethoxymethyl-d4
Molecular Formula: $C_{10}H_6D_4O_4ClF_3S$
Molecular Weight: 322.7

Code Name: AE 1392936 (or M2)
(Soil Metabolite)



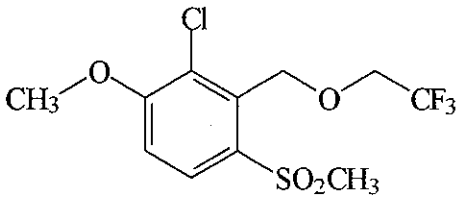
CAS Name: 2-chloro-3-hydroxymethyl-4-mesylbenzoic acid
Molecular Formula: $C_9H_9O_5ClS$
Molecular Weight: 264.69

Code Name: AE 1392936-*benzyl-methylsulfonyl*-d_5
(Soil Metabolite, Deuterated Internal Standard)



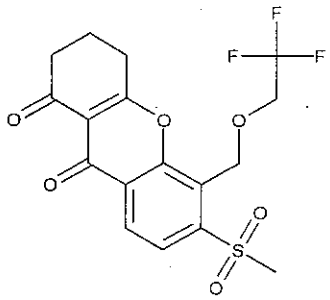
CAS Name: 2-chloro-3-(hydroxymethyl)-4-mesylbenzoic acid-d_5
Molecular Formula: $C_9H_4D_5O_5ClS$
Molecular Weight: 269.70

Code Name: AE 1124336 (or M7)
(Soil Metabolite)



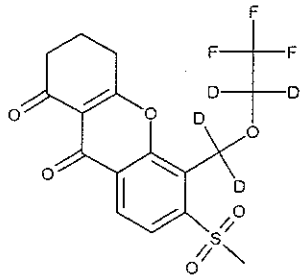
CAS Name: 2-chloro-4-(methylsulfonyl)-1-methoxy-3-[(2,2,2-trifluoroethoxy)methyl]benzene
Molecular Formula: C_{11} H_{12} Cl F_3 O_4 S
Molecular Weight: 332.72

Code Name: AE 0941989 (or M3)
(Soil Metabolite)



CAS Name: 6-(Methylsulfonyl)-5-[(2,2,2-trifluoroethoxy)methyl]-3,4-dihydro-1H-xanthene-1,9(2H)-dione
Molecular Formula C_{17} H_{15} F_3 O_6 S
Molecular Weight: 404.36

Code Name: AE 0941989-*trifluoroethoxymethyl*-d_4
(Soil Metabolite, Deuterated Internal Standard)

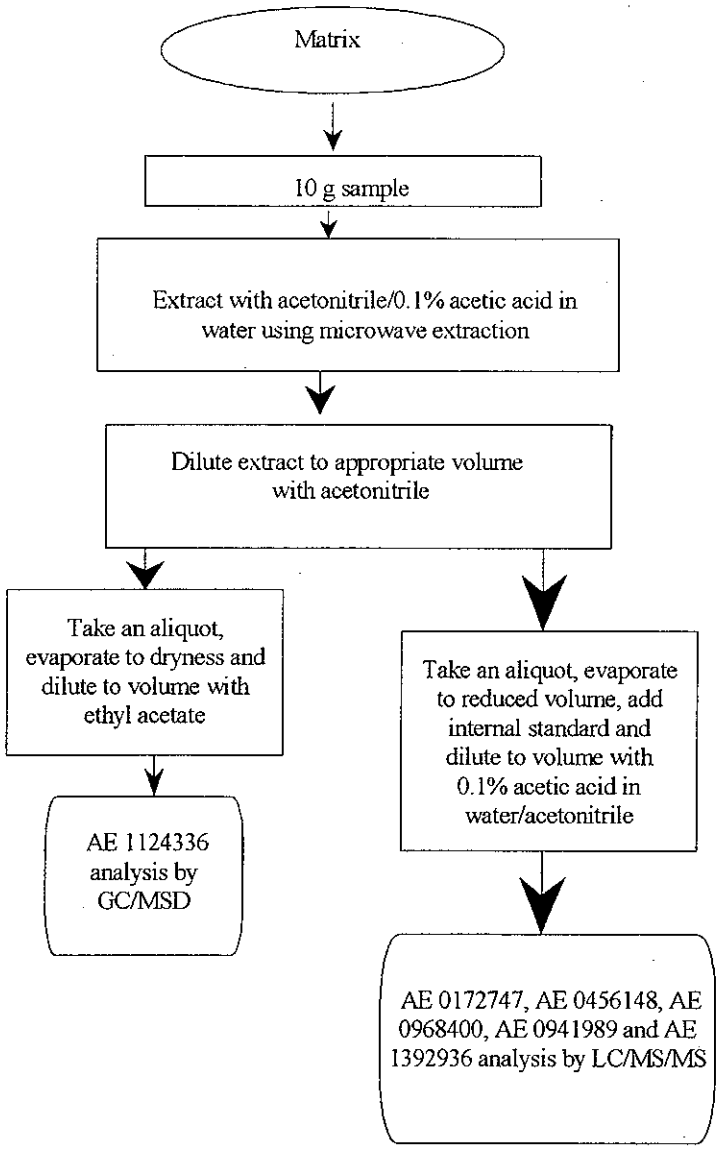


CAS Name: 6-(Methylsulfonyl)-5-[(2,2,2-trifluoroethoxy)methyl]-3,4-dihydro-1H-xanthene-1,9(2H)-dione-trifluoroethoxymethyl-d_4

Molecular Formula: $C_{17}H_9D_4F_3O_6S$

Molecular Weight: 408.39

Appendix 6 Method Flow Chart



Appendix 7 Revision History

Method #	Revision	Description
AE002-S004-02	02	Method prepared on completion of validation study[1]
AE002-S004-03	03	Incorporation of results from ILV[3]