Method Validation Study for the Determination of Residues of X11393728 (XDE-729 Methyl), X11393729 (XDE-729 Acid) and X11449757 (des-Methyl XDE-729 Acid) in Soil using High Performance Liquid Chromatography with Positive-Ion Electrospray Ionization Mass Spectrometry Detection

INTRODUCTION

Scope

This method is applicable for the quantitative determination of residues of XDE-729 methyl, and its major metabolites, XDE-729 acid and des-methyl XDE-729 acid (X‘757) in soil. The method was validated over the concentration range of 0.050-8.0 ng/g with a validated limit of quantitation of 0.050 ng/g in soil. Common and chemical names, molecular formulas, and the nominal masses for the analyte and related compounds are given in Table 1.

This study was conducted to fulfill data requirements outlined in the U. S. EPA Residue Chemistry Test Guidelines, OPPTS 850.7100 (*1*), the European Commission Guidance Document on Residue Analytical Methods, SANCO/825/00 rev. 8.1 (*2*) and SANCO/3029/99 rev.4 (*3*), and PMRA Residue Chemistry Guidelines as Regulatory Directive Dir 98-02 (*4*).

Method Principle

Residues of XDE-729 methyl and its major soil metabolites are extracted from soil by accelerated solvent extraction (ASE), two cycles at 90 °C and 1500 – 2500 psi for 5 minutes with methanol/water (50:50) solution containing 0.1% phosphoric acid. A 5.0 mL aliquot of the extraction solution is placed in a 45-mL vial and a stable isotope internal standard mixture is added. The extract is evaporated to approximately 1 mL and 100 µL of acetonitrile is added. The sample is briefly sonicated and vortex mixed, followed by filtration prior to analysis. The sample is analyzed by liquid chromatography with positive-ion electrospray ionization tandem mass spectrometry (LC-MS/MS).

Safety Precautions

Each analyst must be acquainted with the potential hazards of the equipment, reagents, products, solvents, and procedures used in this method before commencing laboratory work. Sources of information include: operation manuals, Material Safety Data Sheets, literature and other related date. Safety information should be obtained from the supplier. Disposal of waste materials, reagents, reactants, and solvents must be in compliance with applicable governmental requirements.

Acetonitrile, methanol and isopropanol are flammable and should be used in well-ventilated areas away from ignition sources. Phosphoric and formic acid are corrosive and can cause severe burns. It is imperative that proper eye and personal protection equipment be worn when handling these reagents.

Test Substance/Analytical Standard and Internal Standard Reagents

Test Substance/ Analytical Standard	TSN Number	Percent Purity	Certification Date	Reference
X11393728[a] (XDE-729 methyl)	031117-0005	99.1%	26-Jun-2012	FAPC12-000270
X11393729[a] (XDE-729 acid)	030751-0004	99.0%	03-Mar-2011	FAPC10-278379
X11449757[a] (des-methyl-XDE-729 acid)	031413-0001	91%	18-Jan-2012	FAPC11-000348
XR-728-UL ^{13}C phenyl[b] [M+6] stable isotope	301413	99%	26-Jul-2011	FAPC11-000133
XR-729-UL ^{13}C phenyl[b] [M+6] stable isotope	301414	100%	25-Jul-2011	FAPC11-000134
X‘757-UL ^{13}C phenyl[b] [M+6] stable isotope	301486	99%	26-Jul-2011	FAPC11-000147

[a] Test SubstancesAnalytical standard.
[b] Internal standard reagent.

The above reagents and standards may be obtained from the Test Substance Coordinator, Dow AgroSciences LLC, 9330 Zionsville Road, Building 304, Indianapolis, IN 46268-1054. Certificates of Analysis (CoA) for the test substances are found in Figure 1 - Figure 3.

Equipment, Glassware, and Materials

Equipment, glassware, materials, reagents, and chemicals considered to be equivalent to those specified may be substituted with the understanding that their performance must be confirmed by appropriate tests. Common laboratory glassware and supplies are assumed to be readily available. Unless specified otherwise, class A volumetric glassware is recommended to prepare analytical standards, fortification solutions, and calibration standards.

Laboratory Equipment

Accelerated Solvent Extractor, ASE350 or ASE200, Dionex Corporation.

Balance, analytical, Model AE100, Mettler-Toledo, Inc.

Balance, pan, Model BB2440, Mettler-Toledo, Inc.

Bath, ultrasonic, Fisher Scientific.

Evaporator, TurboVap LV, Biotage.

Pipet, positive-displacement, 3-25 µL capacity, Rainin.

Pipet, positive-displacement, 20-50 µL capacity, Rainin.

Pipet, positive-displacement, 50-250 µL capacity, Rainin.

Pipet, positive-displacement, 100-1000 µL capacity, Rainin.

Vortex mixer, Scientific Industries, Inc.

Chromatographic System

Column, analytical, Synergi Hydro-RP 80A, 4.6 mm x 75 mm, 4.0-µm particle size, Phenomenex.

Column, guard, SecurityGuard Cartridge, AQ C18 3.0 mm x 4.0 mm, Phenomenex.

Liquid chromatograph, high performance (HPLC), Aglient 1200 Infinity, Aglilent Technologies.

Mass spectrometer, Model API QTRAP 5500, AB Sciex.

Mass spectrometer data system, Model Analyst 1.5.1, AB Sciex.

Glassware and Materials

Bottle, coated glass, 2-L capacity, Dionex Corporation.

Bottle, glass media, 500-mL capacity, Fisher Scientific.

Cells (ASE350), extraction, stainless steel, 10-mL capacity, Dionex Corporation.

Cells (ASE200), extraction, stainless steel, 11-mL capacity, Dionex Corporation.

Cylinder, graduated, class-B, 50-mL capacity, Fisher Scientific.

Cylinder, graduated, class-B, 500-mL capacity, Fisher Scientific.

Cylinder, graduated, class-B, 1-L capacity, Fisher Scientific.

Cylinder, mixing, class-B, 1-L capacity, Fisher Scientific.

Cylinder, mixing, class-B, 2-L capacity, Fisher Scientific.

Diatomaceous earth (DE), Dionex Corporation.

Flask, volumetric, class-A, 25-mL capacity, Fisher Scientific.

Flask, volumetric, class-A, 50-mL capacity, Fisher Scientific.

Flask, volumetric, class-A, 100-mL capacity, Fisher Scientific.

Flask, volumetric, class-A, 200-mL capacity, Fisher Scientific.

Filters, cellulose, 27-mm, Dionex Corporation.

Filters, cellulose, 19-mm, Dionex Corporation.

Filters, Whatman Mini-Uniprep syringeless, PFTE membrane, 0.45 µm, Fisher Scientific.

Pipet, disposable transfer, Fisher Scientific.

Pipet, volumetric, class-A, 2-mL volume, Fisher Scientific.

Pipet, volumetric, class-A, 5-mL, Fisher Scientific.

Pipet, volumetric, class-A, 10-mL, Fisher Scientific.

Pipet tip, positive-displacement, 25-µL capacity, Rainin.

Pipet tip, positive-displacement, 50-µL capacity, Rainin.

Pipet tip, positive-displacement, 250-µL capacity, Rainin.

Pipet tip, positive-displacement, 1000-µL capacity, Rainin.

Tubes, centrifuge, 15-mL capacity, Fisher Scientific.

Vial, autosampler, 2-mL, National Scientific Company.

Vial, ASE collection, 60-mL, Dionex Corporation.

Vial, 45-mL, Kimble Glass Co.

Vial cap, for autosampler vial, National Scientific Company.

Reagents

Acetonitrile, Chromasolv, Sigma-Aldrich.

Formic acid, ~98%, Sigma-Aldrich.

Isopropanol, HPLC grade, Fisher Scientific.

Methanol, Chromasolv, Sigma-Aldrich.

Phosphoric acid, 85 wt. % in water, Sigma-Aldrich.

Water, Chromasolv, Sigma-Aldrich.

Prepared Solutions

Acetonitrile containing 0.1% formic acid (v/v) (mobile phase B)

Pipet 2.0 mL of formic acid into a 2000-mL graduated mixing cylinder, or equivalent, containing approximately 1900 mL of acetonitrile. Dilute to volume with acetonitrile. Cap the cylinder and invert it multiple times to mix well prior to use. Transfer to appropriate HPLC media bottle. The following alternative method can be used to avoid contamination; however, the concentration of formic acid is approximate rather than exactly 0.1%. Transfer 4.0 mL (4x1.0mL ampules) of formic acid to a freshly opened bottle of acetonitrile. Cap and invert multiple times to mix well prior to use, and then attach 4-L bottle directly to HPLC. Different volumes of solution can be prepared while keeping the ratio between the formic acid and acetonitrile in the same proportions as described above.

Acetonitrile/water/formic acid (10/90/0.1) (v/v/v) (Standard Dilution Solution)

Measure 40.0 mL of acetonitrile using a 50-mL graduated cylinder and transfer into 500-mL media bottle. Measure 360. mL water using a 500-mL graduated cylinder and combine with the acetonitrile in a 500-mL bottle. Add 400. µL of formic acid to the same 500-mL media bottle containing water and acetonitrile. Cap the cylinder and invert several times to mix. Different volumes of solution can be prepared while keeping the ratio between the formic acid, water and acetonitrile in the same proportions as described above.

Isopropanol/water (50:50) (v/v) (Flush Port Rinse)

Measure 2000 mL water using a 2000 mL graduated cylinder and transfer to a clean 4-L solvent bottle. Measure 2000 mL isopropanol using a 2000 mL graduated cylinder and transfer to the same 4-L solvent bottle containing the 2000 mL of water. Cap and invert several times to mix, allow to equilibrate to room temperature. Different volumes of solution can be prepared while keeping the ratio between the isopropanol and water in the same proportions as described above.

Methanol/water (65/35) (v/v) (Standard Dilution Solution)

Measure 300. mL of methanol using a 500-mL graduated cylinder and transfer to 500-mL media bottle. Measure 160. mL of water using a 250-mL graduated cylinder and transfer to 500-mL bottle containing the methanol. Cap the bottle and invert several times to mix. Allow to equilibrate to room temperature. Different volumes of solution can be prepared while keeping the ratio between the methanol and water in the same proportions as described above.

Methanol/water (50/50) containing 0.1% phosphoric acid (v/v) (Extraction Solution)

Measure 1000 mL methanol with graduated cylinder and transfer to 2000-mL plastic coated glass bottle (specific for pressurized extractions). Measure 1000 mL water with a graduated cylinder and transfer to the same 2000-mL bottle containing the methanol. Pipet 2.0 mL of phosphoric acid into the same 2000-mL bottle containing the methanol and water. Cap the bottle and invert it several times to mix well prior to use. Different volumes of solution can be prepared while keeping the ratio between the methanol, water and phosphoric acid in the same proportions as described above.

Water containing 0.1% formic acid (v/v) (mobile phase A)

Pipet 2.0 mL of formic acid into a 2000-mL graduated mixing cylinder containing approximately 1900 mL of water. Dilute to volume with water. Cap the cylinder and invert it multiple times to mix well prior to use. Transfer to the appropriate HPLC media bottle. The following alternative method can be used to avoid contamination; however, the concentration of formic acid is approximate rather than exactly 0.1%. Transfer 4.0 mL (4x1.0mL ampules) of formic acid to a freshly opened bottle of water. Cap and invert multiple times to mix well prior to use, and then attach 4-L bottle directly to HPLC. Different volumes of solution can be prepared while keeping the ratio between the formic acid and water in the same proportions as described above.

EXPERIMENTAL

Instrumental Conditions

Typical HPLC Operating Conditions

Instrumentation:	Agilent 1200
Analytical Column:	Phenomenex, Synergi 4.0-µm Hydro-RP 80Å (75x4.6mm id)
Guard Column:	Phenomenex, SecurityGuard AQ C18 (3.0x4mm)
Column Temperature:	Ambient
Injection Volume:	30 µL
Injection Wash Program	Flush port, 10 seconds

Run Time: ~14 minutes

Mobile Phase: A –Water containing 0.1% formic acid
B –Acetonitrile containing 0.1% formic acid

Flow Rate: 1.0 mL/min.

Mobile Phase Split: Approximately 200 µL /min split to source

Gradient:

Time, min	A, %	B, %
0.00	90	10
7.00	0	100
9.00	0	100
9.15	90	10
13.00	90	10

Flow Diverter Program: 1) 0.0→1.0 min: flow to waste
2) 1.0→10.0 min: flow to source
3) 10.0→end of run: flow to waste

Typical Mass Spectrometry Operating Conditions

Instrumentation: AB SCIEX API 5500 QTrap LC-MS/MS System
AB SCIEX Analyst 1.5.1 data system
Interface: Electrospray
Polarity: Positive
Scan Type: MRM
Resolution: Q1 – unit, Q3 – unit
Curtain Gas (CUR): 40 psi
Collision Gas (CAD): Medium
Temperature (TEM): 550 °C
Ion Source Gas 1 (GS1): 50 psi
Ion Source Gas 2 (GS2): 50 psi

Period 1
Relative Start Time: 0.0 minutes
Duration: 5.0 minutes
Settling Time: 0 ms
MR Pause: 5 ms
Entrance Potential: 10 volts

Compound:

	Ion, *m/z*		Dwell	DP/CE/CXP
	Q1	Q3	Time (ms)	(volts)
X11449757 quantitation	316.73	236.10	125	86/45/32
X11449757 confirmation	318.92	237.90	125	71/45/32
X11449757 [M+6] IS	322.81	242.00	125	56/45/32
XDE-729 (acid) quantitation	330.78	250.00	100	76/41/32
XDE-729 (acid) confirmation	330.78	284.90	100	76/29/40
XDE-729 [M+6] IS	336.86	256.10	100	81/41/34

Period 2

Relative Start Time:	5.0 minutes
Duration:	3.0 minutes
Settling Time:	0 ms
MR Pause:	5 ms
Entrance Potential:	10 volts

Compound:

	Ion, *m/z*			
	Q1	Q3	Time (ms)	DP/CE/CXP
XDE-729 methyl quantitation	344.78	250.00	150	51/43/32
XDE-729 methyl confirmation	344.78	285.00	150	51/31/38
XDE-729 methyl [M+6] IS	350.79	256.10	150	86/43/34

Full-scan and product-ion mass spectra for XDE-729 methyl, XDE-729 (acid) and X11449757 are shown in Figure 4 - Figure 6, respectively.

Typical calibration curves for the determination of XDE-729 methyl, XDE-729 (acid) and X11449757 are shown in Figure 7 - Figure 12, respectively.

Typical chromatograms of calibration standards used in the determination of XDE-729 methyl, XDE-729 (acid) and X11449757 are shown in Figure 13, Figure 14 and Figure 15, respectively. Typical chromatograms for the determination of XDE-729 methyl in a sand, clay loam, silt loam and clay sample are illustrated in Figure 16 - Figure 22. Typical chromatograms for the determination of XDE-729 (acid) in a sand, clay loam, silt loam and clay sample are illustrated in Figure 23 - Figure 26. Typical chromatograms for the determination of X11449757 in a sand, clay loam, silt loam and clay sample are illustrated in Figure 27 - Figure 30. Typical chromatograms of reagent blanks used in the determination of XDE-729 methyl, XDE-729 (acid) and X11449757 are shown in Figure 31, Figure 32 and Figure 33, respectively.

Preparation of Standard Solutions

Preparation of XDE-729 Methyl, XDE-729 Acid and X11449757 Stock Solutions

Weigh 0.0100 g of X11393728 (XDE-729 methyl) analytical standard and quantitatively transfer to 100-mL volumetric flask with acetonitrile. Dilute to volume with acetonitrile to obtain a 100. µg/mL stock solution.

Weigh 0.0100 g of X11393729 (XDE-729 acid) analytical standard and quantitatively transfer to 100-mL volumetric flask with acetonitrile. Dilute to volume with acetonitrile to obtain a 100. µg/mL stock solution.

Weigh 0.0040 g of X11449757 (des-methyl XDE-729 acid) analytical standard and quantitatively transfer to a 200-mL volumetric flask with the 65:35 methanol:water solution. Dilute to volume with the 65:35 methanol:water solution to produce 20.0 µg/mL stock solution.

Pipet 2.0 mL of each of the 100-µg/mL XDE-729 methyl and XDE-729 acid stock solutions and 10.0 mL of the X11449757 stock solution prepared above into a single 100-mL volumetric flask and dilute to volume with the acetonitrile containing 0.1% formic acid solution to obtain a mixed solution containing 2.00 µg/mL of each compound.

Preparation of XDE-729 Methyl, XDE-729 Acid and X11449757 Spiking Solutions

Pipet 5.0 mL of 2.00 µg/mL mixed stock solution prepared above into a 50-mL volumetric flask and dilute to volume with the acetonitrile containing 0.1% formic acid solution to obtain a mixed spiking solution containing 0.20 µg/mL of each compound.

Pipet 5.0 mL of 0.20 µg/mL mixed spiking solution prepared above into a 50-mL volumetric flask and dilute to volume with the acetonitrile containing 0.1% formic acid solution to obtain a mixed spiking solution containing 0.020 µg/mL of each compound.

Pipet 5.0 mL of 0.020-µg/mL mixed spiking solution prepared above into a 50-mL volumetric flask and dilute to volume with the acetonitrile containing 0.1% formic acid solution to obtain a mixed solution containing 0.002 µg/mL of each compound.

Concentration of Spiking Soln.	Aliquot of Spiking Soln.	Equivalent Sample Conc.[a]
µg/mL	µL	ng/g
0.002	50.0	0.020
0.002	125.	0.050
0.020	25.0	0.10
0.20	200.	8.0

[a] The equivalent sample concentration is based on fortifying a 5.0-g soil sample.

Preparation of the Mixed Staple-Isotope Internal Standard Solution

Prepare a 10 µg/mL stock solution of X11278577 (XDE-729 methyl M+6) stable isotope internal standard in acetonitrile. An example would be to weigh 0.0010 g of the X11278577 (XDE-729 methyl M+6) stable-isotope internal standard and quantitatively transfer to a 100-mL volumetric flask with acetonitrile. Dilute to volume with acetonitrile to obtain a 10 µg/mL stock solution.

Prepare a 10 µg/mL stock solution of X12278779 (XDE-729 M+6) stable isotope internal standard in acetonitrile. An example would be to weigh 0.0010 g of the X12278779 (XDE-729 M+6) stable-isotope internal standard and quantitatively transfer to a 100-mL volumetric flask with acetonitrile. Dilute to volume with acetonitrile to obtain a 10 µg/mL stock solution.

Prepare a 10 µg/mL stock solution of X12281837 (X'757 M+6) stable isotope internal standard in the 65:35 methanol:water solution. An example would be to weigh 0.0010 g of the X12281837 (X'757 M+6) stable-isotope internal standard and quantitatively transfer to a 100-mL volumetric flask with the 65:35 methanol:water solution. Dilute to volume with the 65:35 methanol:water solution to obtain a 10 µg/mL stock solution.

Pipet 125 µL each of the 10 µg/mL internal standard stock solutions from above into a single 100-mL volumetric flask and dilute to volume with the acetonitrile:water:formic acid (10:90:0.10) solution to obtain a mixed solution containing nominally 0.0125 µg/mL of each stable isotope internal standard.

Preparation of XDE-729 Methyl, XDE-729 (Acid) and X11449757 Calibration Standards for Samples

Prepare calibration standard solutions by adding 1.0 mL of the 0.0125 µg/mL mixed stable isotope standard solution prepared above and diluting aliquots of the 0.002, 0.020, 0.20 and 2.00 µg/mL mixed solutions with the acetonitrile:water:formic acid (10:90:0.10) solution according to the table below.

Concentration of Spiking Soln.	Aliquot of Spkg. Soln.	Final Soln. Volume	Calibration Soln. Final Conc.	Equiv. Sample Conc.
µg/mL	µL	mL	ng/mL	ng/g[a]
0.002	250	25.0	0.020	0.020
0.002	625	25.0	0.050	0.050
0.020	125	25.0	0.10	0.10
0.020	250	25.0	0.20	0.20
0.20	125	25.0	1.0	1.0
0.20	375	25.0	3.0	3.0
0.20	750	25.0	6.0	6.0
2.00	125	25.0	10.	10.

[a] Equivalent sample concentrations in this table are based on 5.0 gram sample, 25.0 mL extract, 5.0 mL aliquot and 1.0 mL final volume.

Preparation of a XDE-729 Methyl, XDE-729 (Acid) and 11449757 Standards to Determine Isotopic Crossover

Using a positive-displacement pipet, dispense 25 µL of the 0.020 µg/mL mixed spiking solution into an injection vial and using a positive-displacement pipet, dispense 975 µL of the water/acetonitrile/formic acid (90:10:0.1, v/v/v) solution into the same injection vial. Cap the vial and invert several times to mix. The resulting solution contains 0.50 ng/mL of each compound.

Preparation of a XDE-729 Methyl M+6 (X11278577), XDE-729 (Acid) M+6 (X12278779) and X11449757 M+6 (X12281837) Mixed Internal Standards to Determine Isotopic Crossover

Using a positive-displacement pipet, dispense 40 µL of the 0.0125 µg/mL mixed internal standard solution into an injection vial and using a positive-displacement pipet, dispense 960 µL of the water/acetonitrile/formic acid (90:10:0.1, v/v/v) solution into the same injection vial. Cap the vial and invert several times to mix. The resulting solution contains 0.50 ng/mL of each internal standard compound.

The above crossover standards should be stored refrigerated.

Sample Origin, Numbering, Preparation and Storage

Untreated soil control samples were obtained from the Dow AgroSciences Soil Database. All samples were tracked in the Dow AgroSciences LLC Regulatory Labs Information Management System (RLIMS) database. Unique sample numbers were assigned to the samples to track them during receipt, storage, and analysis. Complete source documentation is included in the study file.

The bulk samples were prepared by sieving with a 2mm screen, and stored refrigerated. An aliquot of the sample was removed, placed in a fresh high-density polyethylene (HDPE)

container, logged into RLIMS and designated as a control soil sample for this study. During the course of the study, the samples were stored in temperature-monitored freezers at approximately -20 °C, except when removed for analysis. Physicochemical characteristics for the four soils used are listed in Table 2. Complete documentation for the soil sources are found in the raw study file.

Analysis Procedure

Sample Analysis of XDE-729 methyl, XDE-729 acid and X11449757 by LC-MS/MS

1. Measure 5.0 ±0.05 g portions of sample into 15-mL graduated centrifuge tubes.

2. For preparing fortified samples, add an appropriate volume aliquot of the appropriate spiking solutions to encompass the necessary concentration range:

Concentration of Fortified Sample (ng/g)	Volume of Spiking Solution (µL)	Concentration of Spiking Solution (µg/mL)
0.020	50.0	0.002
0.050	125	0.002
0.10	25.0	0.020
8.0	200	0.20

3. Supplement the soil with diatomaceous earth (DE) to reach approximately the 10-mL mark on tube, cap and shake to thoroughly mix the soil and DE.

4. Prepare extraction cell by placing either a 19.8-mm or 27-mm cellulose filter in the bottom cap of the 11-mL (ASE200) or 10-mL (ASE350) extraction cell, respectively. Add sample to prepared cell and top off with DE, seal the cell tightly then place on cell rack on ASE.

5. Load ASE method:

   a) Preheat = 0 minutes
   b) Heat = 5.0 minutes
   c) Static = 5.0 minutes
   d) Flush [a] = 100%
   e) Purge = 60 seconds
   f) Cycles = 2
   g) Pressure = 1500 psi (or 2500 psi for ASE200)
   h) Temperature = 90 C
   i) Solution X[b] = 100%

[a] Very important to adjust flush percentage to obtain as close to 25 mL of extraction as possible.
[b] Dependent on position placement of extraction solution on system tray.

6. Place labeled collection vials in collection rack and start ASE.

7. Upon completion of ASE extraction (approximately 25 minutes/sample), top off extraction volume to 25.0 mL by placing vial containing extract between two collection vials containing 25.0 mL of extraction solution (check vials) and dilute sample to volume with the methanol:water (50:50) containing 0.1% phosphoric acid solution using the check vials to gauge the volume of your samples. Cap and shake sample gently to mix.

9. Take a 5.0 mL aliquot of the extract and transfer to an 11 dram glass vial.

11. Add 40.0 µL of the 0.0125 µg/mL mixed internal standard solution and gently swirl to mix.

12. Evaporate extract to approximately 1 mL on a TurboVap evaporator set at 40 °C and a nitrogen pressure of approximately 7 psi.

13. Add 100 µL of acetonitrile to each sample.

14. Cap the sample vial with a PTFE-lined cap and sonicate for approximately 10-20 seconds, then vortex for approximately 10-20 seconds.

15. Transfer approximately 500 µL to a 45- µm syringeless filter vial and filter.

16. Analyze the samples and calibration standards for determination of XDE-729 methyl, XDE-729 (acid) and X11449757 by LC-MS/MS with positive-ion electrospray (ESI) tandem mass spectrometry. Determine the suitability of the chromatographic system using the following performance criteria:

    a. Standard curve linearity: Determine that the correlation coefficient equals or exceeds 0.995 for the least squares equation which describes the detector response as a function of standard curve concentration.

    b. Peak resolution: Visually determine that sufficient resolution has been achieved for the analyte and internal standard relative to background interferences.

    c. Appearance of chromatograms: Visually determine the chromatograms with respect to peak response, baseline noise, and background interference. Visually determine that a minimum signal-to-noise ratio of 10:1 has been attained for each analyte in the 0.050-ng/mL calibration standard (equivalent to a sample at the LOQ).

Determination of Isotopic Crossover

In this assay, the analytes and internal standards are quantitated using MS/MS transitions characteristic of each compound. When using stable-isotope labeled internal standards, there is a

possibility that isotopic contributions will occur between the transitions used for quantitation of the unlabeled and labeled compounds. This isotopic overlap between the analyte and the internal standard is determined empirically by analyzing standard solutions prepared separately for each compound and addressed any crossover contributions for accurate determination of concentrations. (*5*, *6*)

To determine the isotopic crossover for each analyte and its respective stable isotope internal standard, inject the crossover standards described in the Preparation of Standard Solutions section, and determine the peak areas for the analytes and internal standards as indicated below.

XDE-729 methyl (X11393728)	*m/z* Q1/Q3 345/250
XDE-729 methyl (M+6) (X11278577)	*m/z* Q1/Q3 351/256
XDE-729 acid (X11393729)	*m/z* Q1/Q3 331/250
XDE-729 acid (M+6) (X12278779)	*m/z* Q1/Q3 337/256
Des-methyl XDE-729 acid (X11449757)	*m/z* Q1/Q3 317/236
Des-methyl XDE-729 acid (M+6) (X12281837)	*m/z* Q1/Q3 323/242

For example, to determine the contribution of the unlabeled XDE-729 methyl to XDE-729 methyl M+6 stable isotope (X11278577), use the XDE-729 methyl standard crossover data from Figure 31 for the quantitation ion:

$$\text{Crossover Factor (analyte}\rightarrow\text{ISTD)} = \frac{\text{peak area of internal standard transition}}{\text{peak area of analyte transition}}$$

$$\text{Crossover Factor (analyte}\rightarrow\text{ISTD)} = \frac{\text{peak area at } m/z \text{ 351/256}}{\text{peak area at } m/z \text{ 345/250}}$$

$$\text{Crossover Factor (analyte}\rightarrow\text{ISTD)} = \frac{0}{357407} = 0$$

In this example, there was no crossover contribution observed from the unlabeled XDE-729 methyl to XDE 729 methyl M+6 stable isotope.

In a similar manner, to determine the contribution of the XDE-729 methyl M+6 stable isoptope

(X11278577) internal standard to the unlabeled XDE-729 methyl using the internal standard crossover data from Figure 31 for the quantitation ion:

$$\text{Crossover Factor (ISTD}\rightarrow\text{analyte)} = \frac{\text{peak area of analyte transition}}{\text{peak area of internal standard transition}}$$

$$\text{Crossover Factor (ISTD}\rightarrow\text{analyte)} = \frac{\text{peak area at } m/z \text{ 345/250}}{\text{peak area at } m/z \text{ 351/256}}$$

$$\text{Crossover Factor (ISTD}\rightarrow\text{analyte)} = \frac{635}{363222} = 0.00175$$

The calculated crossover factor was 0.00175.

To calculate the crossover factors for XDE-729 acid and its XDE-729 acid M+6 stable isotope (X12278779) and des-methyl XDE-729 acid (X11449757) and its X11449757 M+6 stable isotope (X12281837), the same procedures are used as described above.

During method development, the concentration of the labeled internal standards was chosen to minimize the effect of the ISTD→analyte crossover contribution. As a result, no significant mass spectral isotopic crossover was observed and therefore no correction of the measured quantitation ratio was performed. If isotopic crossover is encountered it should be assessed and the respective quantitation ratios corrected for accurate determination of concentrations at the discretion of the study director.

Calculations

Inject the series of calibration standards using the conditions described in the Preparation of Calibration Standard for Samples and determine the peak areas for each analyte and respective internal standard as indicated below.

XDE-729 methyl	*m/z* Q1/Q3 345/250 (quantitation)
	m/z Q1/Q3 345/285 (confirmation)
XDE-729 methyl (M+6)	*m/z* Q1/Q3 351/256 (internal standard)
XDE-729 acid	*m/z* Q1/Q3 331/250 (quantitation)
	m/z Q1/Q3 331/285 (confirmation)
XDE-729 acid (M+6)	*m/z* Q1/Q3 337/256 (internal standard)
X11449757	*m/z* Q1/Q3 317/236 (quantitation)
	m/z Q1/Q3 319/238 (confirmation)
X11449757 (M+6)	*m/z* Q1/Q3 323/242 (internal standard)

For each analyte and standard calculate the quantitation ratio. For example, using the data for the 0.050 ng/mL XDE-729 methyl standard from Figure 16:

$$\text{Quantitation Ratio} = \frac{\text{peak area of analyte transition}}{\text{peak area of internal standard transition}}$$

$$\text{Quantitation Ratio} = \frac{\text{XDE-729 methyl peak area at Q1/Q3 } m/z \text{ 345/250}}{\text{ISTD peak area at Q1/Q3 } m/z \text{ 351/256}}$$

$$\text{Quantitation Ratio} = \frac{45445}{469252} = 0.097$$

Prepare a standard curve for each analyte using linear regression analysis with 1/x weighting by plotting the analyte concentration on the abscissa (x-axis) and the respective quantitation ratio on the ordinate (y-axis) as shown in Figure 7 – Figure 12.

For example, using linear regression (1/x weighting) with the XDE-729 methyl data from Figure 7:

$$Y = (\text{slope} \times X) + \text{intercept}$$

$$X = \frac{Y - \text{intercept}}{\text{slope}}$$

$$\text{XDE-729 methyl (ng/mL)} = \frac{\text{XDE-729 methyl quantitation ratio} - \text{intercept}}{\text{slope}}$$

$$\text{XDE-729 methyl (ng/mL)} = \frac{\text{XDE-729 methyl quantitation ratio} - 0.0057}{1.7835}$$

Calculation of Percent Recovery

Determine the gross concentration in each recovery sample by substituting the corrected quantitation ratio into the above equation and solving for the concentration.

For example, using the data for XDE-729 methyl recovery in sand sample 110716-009-0001A6 + 0.050 ng/g from Figure 34.

$$\text{Quantitation Ratio (sample)} = \frac{7253}{70413} = 0.103$$

$$\text{XDE-729 methyl (gross ng/mL)} = \frac{\text{XDE - 729 methyl quantitation ratio - 0.0057}}{1.7835}$$

$$\text{XDE-729 methyl (gross ng/mL)} = \frac{0.103 - 0.0057}{1.7835}$$

$$\text{XDE-729 methyl (gross ng/mL)} = 0.0546 \text{ ng/mL}$$

Convert the concentration (ng/mL) of XDE-729 methyl found in the final sample prepared for analysis to the concentration (ng/g) of XDE-729 methyl in the original sample as follows:

$$\text{XDE - 729 methyl (gross ng/g)} = \frac{\text{XDE - 729 methyl}}{\text{(gross ng/mL)}} \times \text{MF}$$

Where:

MF = Method Factor

$$\text{MF} = \frac{\text{extraction volume (mL)}}{\text{nominal sample weight (g)}} \times \frac{\text{final volume (mL)}}{\text{aliquot volume (mL)}} = \frac{\text{mL}}{\text{g}}$$

$$\text{MF} = \frac{25 \text{ mL}}{5.0 \text{ g}} \times \frac{1.0 \text{ mL}}{5.0 \text{ mL}} = 1.0 \frac{\text{mL}}{\text{g}}$$

$$\text{XDE - 729 methyl (gross ng/g)} = 0.0546 \text{ ng/mL x } 1.0 \text{ mL/g}$$

$$\text{XDE - 729 methyl (gross ng/g)} = 0.0546 \text{ ng/g}$$

Determine the net concentration in each recovery sample by subtracting any contribution found at the expected retention time of the analyte in the control sample from that of the gross analyte concentration found in the recovery sample.

For example, using the data for XDE-729 methyl from Figure 19 and Figure 34:

$$\text{XDE - 729 methyl (net ng/g)} = \text{XDE - 729 methyl (gross ng/g)} - \text{XDE - 729 methyl (control ng/g)}$$

$$\text{XDE - 729 methyl (net ng/g)} = 0.0546 \text{ ng/g} - 0.0061 \text{ ng/g}$$

$$\text{XDE-729 methyl (net)} = 0.0485 \text{ ng/g}$$

Determine the percent recovery by dividing the net concentration of each recovery sample by the theoretical concentration added.

$$\text{Recovery} = \frac{\text{Concentration Found}}{\text{Concentration Added}} \times 100\%$$

$$\text{Recovery} = \frac{0.0485 \text{ ng/g}}{0.050 \text{ ng/g}} \times 100\%$$

$$\text{Recovery} = 97\%$$

Determination of Soil Moisture in Field Samples

Accurately weigh a 10g portion of soil into a tared aluminum weighing dish.
Place the sample in an oven at 110 °C and allow it to dry for a minimum of 16 hours.
Remove the sample from the oven and place in a dessicator containing Drierite adsorbent.
Re-weigh the sample when it has cooled to room temperature.

The equation to calculate % moisture follows (*7*):

$w = [(W_1 - W_2)/(W_2 - W_c)] \text{ X } 100$

where:

w = moisture content, %

W_1 = weight of container and moist soil

W_2 = weight of container and oven dry soil

W_c = weight of container

Determination of Dry Weight Concentrations of XDE-729 Methyl, XDE-729 (acid) and X11449757 in Soil

Field sample residues are typically reported on a dry-weight basis. The residue concentrations (ng/g) expressed on a dry-weight basis are calculated as follows.

Reported ng/g =) $\frac{\text{p ent( )(oistu(}}{10}$)(

Confirmation of Residue Identity

The method is selective for the determination of XDE-729 methyl, XDE-729 (acid) and X11449757 by virtue of the chromatographic separation and selective detection system used. An additional transition ion was monitored for each analyte for confirmation of its presence. Representative calibration data for the confirmation transitions can be found in Figure 8, Figure 10 and Figure 12 for XDE-729 methyl, XDE-729 (acid) and des-methyl XDE-729 acid respectively. The recovery and precision data for the confirmation transitions are summarized in Table 34 – Table 37. In addition, Figure 34 - Figure 45 show typical chromatograms of both the quantitation transition and the confirmation transitions from untreated sand (M842), clay loam (M830), silt loam (M841) and clay (M837) samples fortified with 0.050 ng/g, 0.10 ng/g and 8.0 ng/g of XDE-729 methyl, XDE-729 (acid) and X11449757.

Statistical Treatment of Data

Statistical treatment of data included the calculation of regression equations, coefficients of determination (r^2) for describing the linearity of calibration curves, and means, standard deviations, relative standard deviations of the results for the fortified recovery samples and the Grubbs‘ test to determine outliers.

Extraction Efficiency

Extensive efforts were undertaken in the aerobic soil metabolism study (*8*) to verify the suitability of the ASE extraction procedure to remove XDE-729 methyl and its metabolites from soil. In the anaerobic soil degradation study (*9*) the suitability of the extract procedures was verified by the ASE extraction of the sterile soils. The non-extractable residues accounted for typically less than 10% of applied radioactivity, indicating that the extraction procedure adequately extracted aged XDE-729 methyl residues, and its metabolites, from soil.

Table 1. Identity and Structures of Analytes and Related Compounds

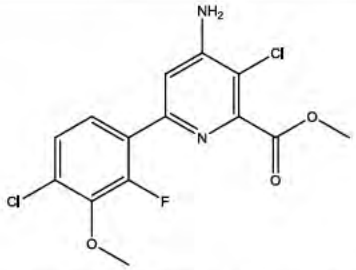
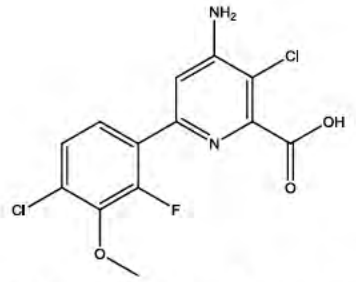
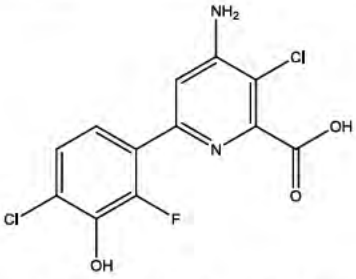
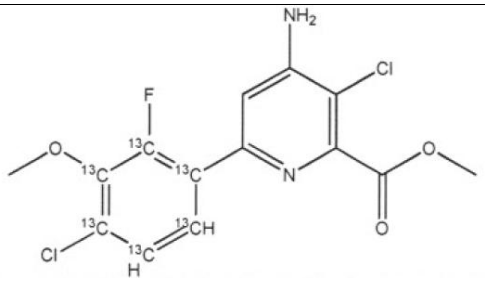
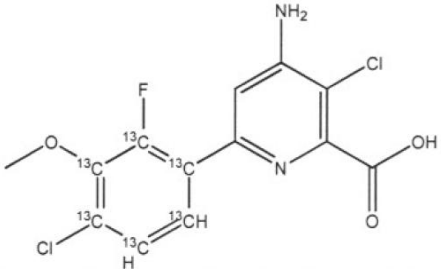
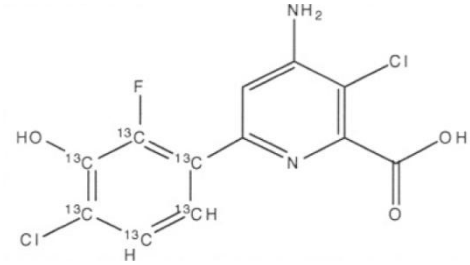
Common Name of Compound		Structural Formula and Chemical Name
XR-729 Methyl, X11393728 Molecular Formula: Formula Weight: Nominal Mass: CAS Number	 $C_{14}H_{11}Cl_2FN_2O_3$ 345.15 345 943831-98-9	 4-amino-3-chloro-6-(4-chloro-2-fluoro-3-methoxy-phenyl)-pyridine-2-carboxylic acid methyl ester
XDE-729 Acid, X11393729 Molecular Formula: Formula Weight: Nominal Mass: CAS Number	 $C_{13}H_9Cl_2FN_2O_3$ 331.13 331 943832-60-8	 4-amino-3-chloro-6-(4-chloro-2-fluoro-3-methoxy-phenyl)-pyridine-2-carboxylic acid
X11449757 Molecular Formula: Formula Weight: Nominal Mass: CAS Number	 $C_{12}H_7Cl_2FN_2O_3$ 317.10 317 not available	 4-amino-3-chloro-6-(4-chloro-2-fluoro-3-hydroxyphenyl)picolinic acid

Table 1 (Cont.) Identity and Structures of Analytes and Related Compounds

Common Name of Compound	Structural Formula and Chemical Name
XR-728-UL ^{13}C Phenyl (XR-729 methyl M+6) stable isotope Molecular Formula: $C_8{}^{13}C_6H_{11}Cl_2FN_2O_3$ Formula Weight: 351.11 Nominal Mass: 351 CAS Number not available	 Methyl 4-amino-3-chloro-6-(4-chloro-3-methoxyphenyl)pyridine-2-carboxylate UL ^{13}C phenyl
XR-729-UL ^{13}C Phenyl (XR-729 acid M+6) stable isotope Molecular Formula: $C_7{}^{13}C_6H_9Cl_2FN_2O_3$ Formula Weight: 337.08 Nominal Mass: 337 CAS Number not available	 4-amino-3-chloro-6-(4-chloro-2-fluoro-3-methoxy-phenyl)-2-pyridine carboxylic acid UL ^{13}C phenyl
X11449757 M+6 stable-isotope Molecular Formula: $C_6{}^{13}C_6H_7Cl_2\ FN_2O_3$ Formula Weight: 323.06 Nominal Mass: 323 CAS Number not available	 4-amino-3-chloro-6-(4-chloro-2-fluoro-3-hydroxy-phenyl)-2-pyridine carboxylic acid UL ^{13}C phenyl