

## Method Validation Study for the Determination of Residues of XDE-729 Methyl and Its Acid Metabolite in Soil by Liquid Chromatography with Tandem Mass Spectrometry

### INTRODUCTION

#### Scope

This method is applicable for the quantitative determination of residues of XDE-729 methyl and XDE-729 acid (quantified as XDE-729 butyl ester) in soil (sandy loam and clay loam). The method was validated over the concentration range of 0.0015-0.1000 ng/g with a validated limit of quantitation of 0.0015 ng/g. Common names, chemical names, and molecular formulas for the analytes are given in Table 1.

This study was conducted to fulfill data requirements outlined in the EPA Residue Chemistry Test Guidelines, OCSPP 850.6100 (1). The validation also complies with the requirements of EU Council Regulation (EC) No. 1107/2009 with particular regard to Section 4 of SANCO/3029/99 rev.4 and Section 5 of SANCO/825/00 rev.8.1 as well as PMRA Residue Chemistry Guidelines as Regulatory Directive Dir98-02 (2-4). The validation was conducted following Dow AgroSciences SOP ECL-24 with exceptions noted in the protocol or by protocol amendment.

#### Method Principle

Residues of XDE-729 methyl and XDE-729 acid are extracted from soil samples by shaking with acetonitrile/water (80/20,v/v) with 0.1% phosphoric acid. The total sample extract is then diluted with water and purified using a strong cation exchange (SCX) solid-phase extraction (SPE) procedure. The SPE eluate is evaporated to dryness and the XDE-729 acid residues are derivatized to form butyl esters to achieve the desired method LOQ and selectivity. The sample is then evaporated and reconstituted in acetonitrile/water containing 0.1% phosphoric acid (70/30, v/v). The final sample is analyzed for XDE-729 methyl and XDE-729 butyl ester by liquid chromatography coupled with positive-ion electrospray tandem mass spectrometry (LC-MS/MS).

#### Test Substances/Reference Compounds/Analytical Standards

Test Substance	TSN	Percent Purity	Re-Certification Date	Reference
XDE-729 Methyl	031117-0005	99.1	21-Nov-16	FAPC14-000293
X11278577	305882	100	16-Nov-16	FAPC14-000255
XDE-729 Acid	030751-0004	99	07-Nov-17	FAPC13-000599
X12278779	305883	100	04-Nov-15	FAPC13-000586

The Certificates of Analysis for the test substances can be found in Figures 1-4. The above standards may be obtained free of charge from Dow AgroSciences LLC, 9330 Zionsville Road, Building 304, Indianapolis, IN 46268-1054.

## EXPERIMENTAL

### Sample Origin, Numbering, Preparation, Storage, and Characterization

Untreated control samples were obtained from the Dow AgroSciences LLC Sample Management Group. 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.

During the course of the study, the samples were stored in temperature-monitored freezers and refrigerators at approximately -20 °C or 4 °C, except when removed for analysis. Physicochemical characteristics for the soils used are listed in Table 2. Complete documentation for the soil sources may be found in the raw study file.

### Determination of Isotopic Crossover

In this assay, the analytes and internal standards (ISTD) 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 of each compound separately at analytically relevant concentrations and if observed, should be addressed for accurate determination of analyte concentrations.

To assess isotopic crossover, a sample was fortified at the working concentration of the internal standard in neat solution that is not spiked with any analytes. In addition, the lowest and highest calibration standards were prepared without internal standards in neat solution. The peak areas for the analytes and internal standards were determined as indicated below.

XDE-729 Methyl	<i>m/z</i> Q1/Q3 345/250 (Quantitative)
XDE-729 Methyl	<i>m/z</i> Q1/Q3 345/235 (Confirmatory)
XDE-729 Methyl	<i>m/z</i> Q1/Q3 351/256 (ISTD, M+6)
XDE-729 Butyl Ester	<i>m/z</i> Q1/Q3 387/250 (Quantitative)
XDE-729 Butyl Ester	<i>m/z</i> Q1/Q3 387/207 (Confirmatory)
XDE-729 Butyl Ester	<i>m/z</i> Q1/Q3 393/256 (ISTD, M+6)

During method development, the concentration range of the calibration curve and the concentration of the labeled internal standard were chosen to minimize the effect of the analyte→ISTD or ISTD→analyte crossover contribution. As a result, no mass spectral isotopic crossover was observed from the analyte to ISTD. In addition, no mass spectral isotopic crossover was observed from the ISTD to the analyte peak area for XDE-729 methyl or XDE-729 butyl ester.

### Calculation of Standard Calibration Curve

Calculation of a standard curve begins with the injection of a series of calibration standards described in Appendix I and acquisition of peak areas for the following analytes.

XDE-729 Methyl	<i>m/z</i> Q1/Q3 345/250 (Quantitative)
XDE-729 Methyl	<i>m/z</i> Q1/Q3 345/235 (Confirmatory)
XDE-729 Methyl	<i>m/z</i> Q1/Q3 351/256 (ISTD, M+6)
XDE-729 Butyl Ester	<i>m/z</i> Q1/Q3 387/250 (Quantitative)
XDE-729 Butyl Ester	<i>m/z</i> Q1/Q3 387/207 (Confirmatory)
XDE-729 Butyl Ester	<i>m/z</i> Q1/Q3 393/256 (ISTD, M+6)

For each analyte, the linearity of detector response was evaluated using solvent calibration standard solutions. In order to generate a standard curve, the analyte concentration was plotted on the abscissa (x-axis) and the respective quantitation ratio on the ordinate (y-axis) in Analyst. Using regression analysis, the equation for the curve was determined with respect to the abscissa. Refer to Figures 5-8 for example calibration plots and to Figures 9 and 10 for example calculations. Calibration data for each analytical set can be found in Tables 3-6.

### Full-Scan and Product-Ion Mass Spectra

A full scan and product-ion mass spectra of XDE-729 methyl and XDE-729 butyl ester are illustrated in Figures 11-14.

### Statistical Treatment of Data

Statistical treatment of data included but was not limited to the calculation of regression equations, correlation coefficients (*r*) for describing the linearity of calibration curves, and means, standard deviations, and relative standard deviations of the results for the fortified recovery samples.

Table 1. Identities and Structures of Analytes and Internal Standards

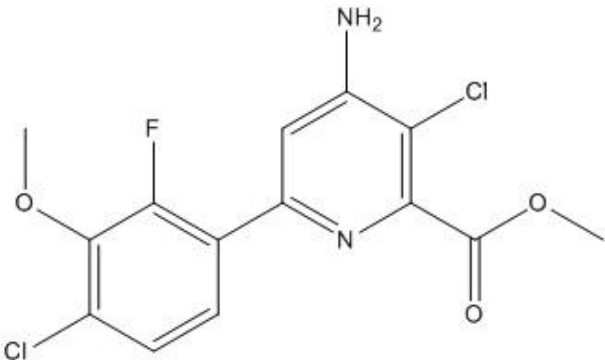
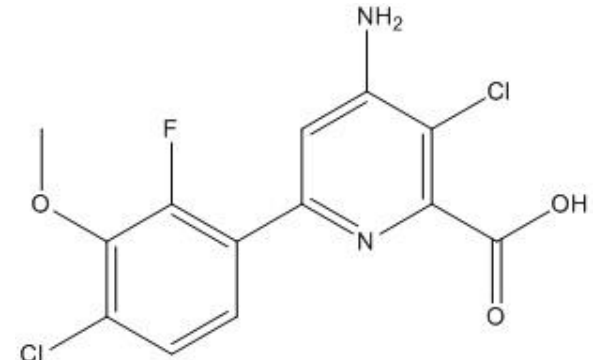
	Structural Formula and Chemical Name
<p>Common Name of Compound: XDE-729 Methyl, XDE-729 Methyl Ester, or Halauxifen-Methyl</p> <p>Molecular Formula: <math>C_{14}H_{11}Cl_2FN_2O_3</math></p> <p>Formula Weight: 345.16</p> <p>CAS Number: 943831-98-9</p>	 <p>4-amino-3-chloro-6-(4-chloro-2-fluoro-3-methoxyphenyl)pyridine-2-carboxylate</p>
<p>Common Name of Compound: XDE-729 Acid or Halauxifen-Acid</p> <p>Molecular Formula: <math>C_{13}H_9Cl_2FN_2O_3</math></p> <p>Formula Weight: 331.13</p> <p>CAS Number: 943832-60-8</p>	 <p>4-amino-3-chloro-6-(4-chloro-2-fluoro-3-methoxyphenyl)pyridine-2-carboxylic acid</p>

Table 1. Cont. Identities and Structures of Analytes and Internal Standards

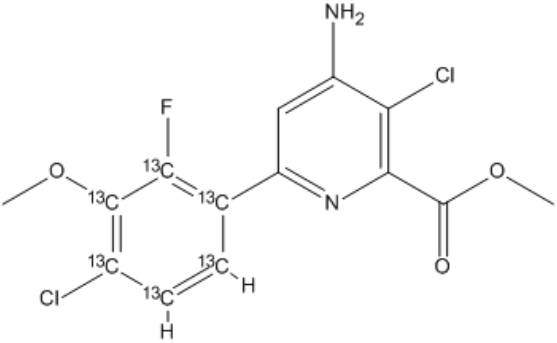
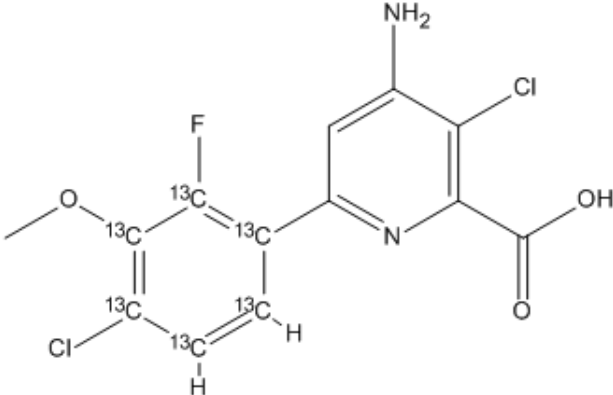
<p>Common Name of Compound:</p> <p>X11278577 or XDE-729 Methyl ISTD, M+6</p> <p>Molecular Formula: <math>C_8^{13}C_6H_{11}Cl_2FN_2O_3</math></p> <p>Formula Weight: 351.11</p> <p>CAS Number: NA</p>	<p><u>Structural Formula and Chemical Name</u></p>  <p>methyl 4-amino-3-chloro-6-(4-chloro-2-fluoro-3-methoxyphenyl)pyridine-2-carboxylate UL 13C phenyl</p>
<p>Common Name of Compound:</p> <p>X12278779 or XDE-729 Acid ISTD, M+6</p> <p>Molecular Formula: <math>C_7^{13}C_6H_9Cl_2FN_2O_3</math></p> <p>Formula Weight: 337.08</p> <p>CAS Number: NA</p>	<p><u>Structural Formula and Chemical Name</u></p>  <p>4-amino-3-chloro-6-(4-chloro-2-fluoro-3-methoxyphenyl)-pyridine-2-carboxylic acid</p>

Table 2. Physicochemical Characteristics of Test Soil

Parameter	Results			
Study Designation	M956 (SGN -001)	M957 (SGN -002)	M958 (SGN -003)	M960 (SGN -004)
Soil Series				
Geographic Location	Brierlow, Derbyshire, UK	Longwoods, Lincolnshire, UK	Schmallenberg, North Rhine, Germany	Stanislaus, California, USA
USDA Texture Class	Silt Loam	Sandy Loam	Loam	Loam
% Sand	39	65	37	43
% Silt	53	25	46	40
% Clay	8	10	17	17
International Texture Class	Sandy Loam	Sandy Loam	Clay Loam	Clay Loam
% Sand	69	83	55	61
% Silt	23	7	28	22
% Clay	8	10	17	17
pH <sup>c</sup>	5.6	7.9	6.1	7
% Organic Carbon	4.5	0.6	5.3	0.7
Microbial Biomass (µg/g)	844.2	348.8	1299.6	755.7
CEC (meq/100g)	23.6	4.6	22.8	8.6
Moisture Holding Capacity at 1/3 bar	32.2%	19.0%	31.2%	21.2%
Bulk Density (g/cm <sup>3</sup> )	0.95	1.29	0.86	1.22

pH Analytical Procedure – 1:1 soil : water Method  
 Organic Carbon determined using furnace method  
 CEC = Cation Exchange Capacity, NH<sub>4</sub> Displacement Method  
 Bulk Density – Disturbed Samples

## Method Validation Study for the Determination of Residues of XDE-729 Methyl and XDE-729 Acid in Soil by Liquid Chromatography with Tandem Mass Spectrometry

### Scope

This method is applicable for the quantitative determination of XDE-729 methyl and XDE-729 acid (quantified as XDE-729 butyl ester) in soil. The method concentration range is 0.0015-0.1000 ng/g with a validated limit of quantitation of 0.0015 ng/g.

### 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 data. Safety information should be obtained from the supplier. Disposal of waste materials, reagents, reactants, and solvents must be in compliance applicable governmental requirements.

Acetonitrile, 1-butanol, butyl chloroformate, pyridine, isopropyl alcohol and methanol are flammable and should be used in well-ventilated areas away from ignition. Ammonium hydroxide, formic acid and phosphoric acid are corrosive and can cause severe burns. It is imperative that proper eye and personal protection equipment be worn when handling these reagents.

### Laboratory Equipment

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

Balance, pan, Model PM2000, Mettler-Toledo, Inc.

Centrifuge, Allegra 6 series, catalog number 366802, Beckman Coulter.

Evaporator, Turbovap LV, model number ZW700, Zymark.

Pipet, positive-displacement, 10-25  $\mu$ L capacity, Model M25, Gilson Inc.

Pipet, positive-displacement, 10-100  $\mu$ L capacity, Model M100, Gilson Inc.

Pipet, positive-displacement, 50-250  $\mu$ L capacity, Model M250, Gilson Inc.

Pipet, positive-displacement, 100-1000  $\mu$ L capacity, Model M1000, Gilson Inc.

Repeater, positive-displacement, 1-25 mL capacity, catalog number 022260201, Eppendorf.

Repeater Stream Pipet, 1-50 mL capacity, catalog number 022460803 Eppendorf.

Shaker, variable speed reciprocating with box carrier, Model 6000, Eberbach Corporation.

Ultrasonic cleaner, Model 1200, Branson Cleaning Equipment Company.

Vacuum manifold, Model spe-12G, Mallinckrodt Baker, Inc.

Vortex mixer, catalog number 02215365, Fisher Scientific.

### Chromatographic System

Column, analytical, Acquity UPLC HSS T3, 2.1 x 50 mm, 1.8 µm particle size, catalog number 186003538, Waters.

Liquid chromatography, 1290 Infinity, Agilent.

Mass spectrometer, Model QTRAP 5500, AB SCIEX.

Mass spectrometer data system, Analyst v.1.6.2, AB SCIEX.

### Glassware and Materials

Cap, 24 mm, PTFE face lined, catalog number 02-912-069, Fisher Scientific.

Centrifuge Tubes, 50 mL catalog number 06-443-20, Fisher Scientific

Column, Strata SCX, 100 mg, 3 mL, catalog number 8B-S010-EBJ, Phenomenex.

Combitips 0.5 mL, catalog number 0030089421, Eppendorf.

Combitips 2.5 mL, catalog number 0030089448, Eppendorf.

Combitips 5 mL, catalog number 0030089456, Eppendorf.

Combitips 10 mL, catalog number 0030089464, Eppendorf.

Filter Aid, 3M Empore, catalog number 14-378-6, Fisher Scientific.

Glass pastuer pipet, 5.75 in. length, catalog number 13-678-6A, Fisher Scientific.

Graduated cylinder, borosilicate glass, 100 mL, catalog number S63458, Fisher Scientific.

Graduated cylinder, borosilicate glass, 500 mL, catalog number S63460, Fisher Scientific.

Graduated cylinder, borosilicate glass, 1000 mL, catalog number S63461, Fisher Scientific.

Pipet tip, positive-displacement, 25 µL capacity, catalog number CP25, Gilson Inc.

Pipet tip, positive-displacement, 100 µL capacity, catalog number CP100, Gilson Inc.

Pipet tip, positive-displacement, 250 µL capacity, catalog number CP250, Gilson Inc.

Pipet tip, positive-displacement, 1000 µL capacity, catalog number CP1000, Gilson Inc.

Vial, autosampler, 1.5 mL, catalog number C4000-V1, National Scientific Company.

Vial, 11-dram, screw thread, catalog number 60958A-11, Kimble Chase.

Vial cap, for autosampler vial, catalog number C4000-55B, National Scientific Company.

Volumetric flask, 10 mL, catalog number K623010-0010, Fisher Scientific.

Volumetric flask, 20 mL, catalog number K623010-0020, Fisher Scientific.

Volumetric flask, 100 mL, catalog number K623010-0100, Fisher Scientific.



## Reagents

1-butanol, catalog number A339-4, Fisher Scientific

Acetonitrile, Chromasolv HPLC grade, catalog number 34851-4L, Sigma Aldrich.

Ammonium Hydroxide, catalog number A669S-500, Fisher Scientific

Butyl chloroformate, catalog number 184462-100G, Sigma Aldrich.

Formic Acid, Optima LC/MS, catalog number A117-50, Fisher Scientific.

Isopropyl Alcohol, OmniSolv, catalog number PX1834-1, EMD

Methanol, Chromasolv HPLC grade, catalog number 34885-4L-R, Sigma Aldrich.

Phosphoric Acid, catalog number A242-500, Fisher Scientific.

Pyridine, anhydrous, catalog number 270970-25mL, Sigma Aldrich.

Water, Chromasolv HPLC grade, catalog number 270733-4L, Sigma Aldrich.

## Prepared Solutions

### *Acetonitrile/Water (80/20, v/v) containing 0.1% Phosphoric Acid (Extraction Solution)*

Measure 800 mL of acetonitrile with a 1 L graduated cylinder and transfer to a 1 L bottle. Measure 200 mL of water with a 250 mL graduated cylinder and transfer to the bottle. Pipette 1 mL of phosphoric acid to the bottle. Cap and mix.

### *Water containing 0.1% Phosphoric Acid (SPE Conditioning and SPE Wash)*

Measure 1 L of water with a 1 L graduated cylinder and transfer to a 1 L bottle. Pipette 1 mL of phosphoric acid to the bottle. Cap and mix.

### *Methanol containing 0.1% Phosphoric Acid (SPE Conditioning and SPE Wash)*

Measure 1 L of methanol with a 1 L graduated cylinder and transfer to a 1 L bottle. Pipette 1 mL of phosphoric acid to the bottle. Cap and mix.

### *Acetonitrile/Methanol (90/10, v/v)*

Measure 900 mL of acetonitrile with a 1 L graduated cylinder and transfer to a 1 L bottle. Measure 100 mL of methanol with a 100 mL graduated cylinder and transfer to the bottle. Cap and mix.

### *Acetonitrile/methanol (90/10, v/v) containing 1% Ammonium Hydroxide (SPE Elution)*

Pipette 1.0 mL of ammonium hydroxide into a 100 mL volumetric flask. Dilute to volume with the acetonitrile/methanol (90/10, v/v) solution. Prepare the solution fresh every two days.

*Acetonitrile/Water (70/30, v/v) containing 0.1% Phosphoric Acid*

Measure 70 mL of acetonitrile with a 100 mL graduated cylinder and transfer to a 4 oz bottle. Measure 30 mL of the water containing 0.1% phosphoric acid solution with a 50 mL graduated cylinder and transfer to the same bottle.

*Acetonitrile/Pyridine/1-Butanol (11/2/1, v/v/v) Coupling Reagent*

Pipette 11 mL of acetonitrile into a 20 mL vial. Pipette 2 mL of pyridine into the same vial. Pipette 1 mL of 1-butanol into the same vial. Cap with a PTFE lined cap and mix. Prepare the solution fresh every two weeks.

*Acetonitrile/Butyl Chloroformate (90/10, v/v) Reagent*

Pipette 4.5 mL of acetonitrile into a 3 dram vial. Pipette 0.5 mL of butyl chloroformate into the same vial. Cap with a PTFE lined cap and mix. Prepare fresh every three days.

*Isopropyl Alcohol/Water (50/50, v/v) (Autosampler Wash)*

Measure 500 mL of isopropyl alcohol with a 500 mL graduated cylinder and transfer to a 1 L bottle. Measure 500 mL of water with a 500 mL graduated cylinder and transfer into the 1 L bottle. Cap and mix.

*Water containing 0.1% Formic Acid (Mobile Phase)*

Measure 1 L of water with a 1 L graduated cylinder and transfer to a 1 L bottle. Pipette 1 mL of formic acid to the bottle. Cap and mix.

*Acetonitrile containing 0.1% Formic Acid (Mobile Phase)*

Measure 1 L of acetonitrile with a 1 L graduated cylinder and transfer to a 1 L bottle. Pipette 1 mL of formic acid to the bottle. Cap and mix.

Note: Allow all solutions to equilibrate to room temperature before use.

### Preparation of Stock and Spiking Solutions

1. Weigh 0.0100 g of XDE-729 methyl (X11393728, Halauxifen methyl) and XDE-729 acid (X11393729, Halauxifen acid) analytical standards and quantitatively transfer to separate 100 mL volumetric flasks with methanol. Dilute to volume with methanol to obtain separate 100 µg/mL stock solutions of each analyte.
2. Pipet 2.0 mL of each of the 100 µg/mL stock solutions into a single 20 mL volumetric flask. Dilute to volume with methanol to obtain a 10 µg/mL mixed spiking solution.
3. Pipet 2.0 mL of the 10 µg/mL mixed spiking solution into a 20 mL volumetric flask and adjust to volume with methanol to obtain a 1.0 µg/mL mixed spiking solution.
4. Pipet 2.0 mL of the 1.0 µg/mL mixed spiking solution into a 20 mL volumetric flask and adjust to volume with methanol to obtain a 0.1 µg/mL mixed spiking solution.
5. Pipet 2.0 mL of the 0.1 µg/mL mixed spiking solution into a 20 mL volumetric flask and adjust to volume with methanol to obtain a 0.01 µg/mL mixed spiking solution.
6. Pipet 2.0 mL of the 0.01 µg/mL mixed spiking solution into a 20 mL volumetric flask and adjust to volume with methanol to obtain a 0.001 µg/mL mixed spiking solution.

### Preparation of Internal Standard Solutions

1. Weigh 0.0020 g of XDE-729 methyl internal standard (X12278577) and XDE-729 acid internal standard (X12278779) and quantitatively transfer to separate 20 mL volumetric flasks with methanol. Dilute to volume with methanol to obtain separate 100 µg/mL stock solutions of each internal standard.
2. Pipet 2.0 mL of each of the 100 µg/mL internal standard stock solutions into a single 20 mL volumetric flask. Dilute to volume with methanol to obtain a 10 µg/mL mixed internal standard solution.
3. Pipet 2.0 mL of the 10 µg/mL internal standard stock solutions into a single 20 mL volumetric flask. Dilute to volume with methanol to obtain a 1.0 µg/mL mixed internal standard solution.
4. Pipet 500 µL of the 1.0 µg/mL internal standard stock solutions into a single 20 mL volumetric flask. Dilute to volume with methanol to obtain a 25.0 ng/mL mixed internal standard solution.
5. Pipet 2.0 mL of the 25.0 ng/mL internal standard stock solutions into a single 20 mL volumetric flask. Dilute to volume with methanol to obtain a 2.5 ng/mL mixed internal standard solution.

### Preparation of Fortification Solutions

Prepare fortification solutions for X11393728 and X11393729 by following the table below. Dilute to volume with methanol.

Initial Stock Solution Concentration (µg/mL)	Aliquot Of Stock Solution (mL)	Final Solution Volume (mL)	Fortification Solution Concentration (ng/mL)
0.100	0.500	10	5.000
0.001	1.500	10	0.150
0.001	0.450	10	0.045

### Preparation of Intermediate Calibration Standards

Prepare intermediate calibration standards in separate 10 mL volumetric flasks by dispensing the appropriate amount of the mixed analyte spiking solution into the flasks. Dilute the intermediate calibration standards to volume with methanol.

Conc. Of Spiking Solution (µg/mL)	Aliquot of Spiking Soln. (ml)	Final Solution Volume (mL)	Calib. Conc. In 10 mL <sup>a</sup> (ng/mL)	Calib. Conc. In 0.5 mL <sup>b</sup> (ng/mL)	Equivalent Sample Conc. <sup>c</sup> (ng/g)
0.100	0.50	10	5.000	2.5000	0.25000
0.100	0.20	10	2.000	1.0000	0.10000
0.100	0.10	10	1.000	0.5000	0.05000
0.010	0.60	10	0.600	0.3000	0.03000
0.010	0.20	10	0.200	0.1000	0.01000
0.010	0.10	10	0.100	0.0500	0.00500
0.001	0.30	10	0.030	0.0150	0.00150
0.001	0.09	10	0.009	0.0045	0.00045

<sup>a</sup>Concentration after diluting to volume with methanol.

<sup>b</sup>Concentration after taking 250 µL aliquot and reconstituting in 500 µL final volume.

<sup>c</sup>The equivalent sample concentration is based on spiking a 5 g sample, 30 mL of extraction solution and 500 µL final sample volume.

Volumes may be scaled to accommodate alternate preparations for the spiking and calibration standard solutions. Refrigeration of solutions is recommended to help reduce evaporation and maintain standard integrity.

Analytical Procedure

1. Weigh  $5.0 \pm 0.05$  g of the sample into a 50 mL centrifuge tube.
2. To prepare fortified samples, add the appropriate aliquot of mixed spiking solution to encompass the concentration range from 0.00045-0.10000 ng/g. For example:

Sample Name	Concentration of Mixed Fortification Solution (ng/mL)	Volume of Fortification Solution ( $\mu$ L)	Concentration of Fortified Sample (ng/g)
Control	N/A	N/A	N/A
LOD	0.045	50	0.00045
LOQ	0.150	50	0.00150
HIGH	5.000	100	0.10000

3. Add 15 mL of acetonitrile/water (80/20, v/v) containing 0.1%  $H_3PO_4$  to the sample bottle.
4. Cap the centrifuge tube and shake the sample for a minimum of 30 minutes on a reciprocating shaker at approximately 280 excursions/minute.
5. Centrifuge for 5 minutes at 3000 rpm.
6. Transfer the supernatant to a 45 mL (11 dram) vial.
7. Add 15 mL of acetonitrile/water (80/20, v/v) containing 0.1%  $H_3PO_4$  extraction solution to the soil sample from step 6 and vortex until soil is mixed from the bottom of the tube.
8. Cap the centrifuge tube and shake the sample for a minimum of 30 minutes on a reciprocating shake at approximately 280 excursions/minute.
9. Centrifuge for 5 minutes at 3000 rpm.
10. Combine the extraction solution from step 9 to the extraction solution from step 6 in the 45 mL vial.
11. Evaporate the organic solvent (until less than 6 mL aqueous remains) using a TurboVap evaporator set at 40°C and a nitrogen pressure of approximately 15 psi.
12. Add 3 mL of HPLC water to the vial containing the evaporated extract. Vortex mix and sonicate for approximately 30 seconds.
13. Purify the sample using the following SPE procedure:
  - a. Place a 100-mg (3 mL) Strata SCX SPE cartridge on the vacuum manifold. Add approximately a 3 mm layer of filter aid to each cartridge.
  - b. Condition the SPE cartridge with 3 mL of methanol containing 0.1%  $H_3PO_4$  followed by 3 mL of water containing 0.1%  $H_3PO_4$ . Dry the cartridge under vacuum for 5-10 seconds between solvents. Discard the eluate.
  - c. Transfer the entire sample from Step 12 to the SPE cartridge. Pull the samples through the cartridge at approximately 0.5 mL/min, discarding the eluate. Dry the cartridge under vacuum for about 5-10 seconds.

- d. Wash the sample vial with 2 mL of water containing 0.1% H<sub>3</sub>PO<sub>4</sub> and transfer to the SPE cartridge. Dry the cartridge under vacuum for about 5-10 seconds. Discard the eluate.
  - e. Wash the SPE cartridge with two 1 mL aliquots of methanol containing 0.1% H<sub>3</sub>PO<sub>4</sub>. Dry the cartridge under full vacuum for about 60 seconds. Discard the eluate.
  - f. Elute the analytes from the SPE cartridge at approximately 1 mL/min with two 1.5 mL aliquots of an acetonitrile/methanol (90/10, v/v) solution containing 1.0% NH<sub>4</sub>OH collecting the eluate in 12-mL culture tubes. Apply full vacuum for about 10 seconds after SPE stops dripping to ensure complete recovery of solvent.
14. Add 15 µL of 2.5 ng/mL mixed internal standard to each sample.
  15. Pipette 250 µL aliquots of the intermediate calibration standards into 12-mL culture tubes followed by 15 µL aliquots of the 2.5 ng/mL mixed IS standard into the same culture tubes.
  16. Evaporate the samples and standards to dryness using a TurboVap evaporator set at 40 °C and a nitrogen pressure of approximately 15 psi.
  17. Add 100 µL of coupling reagent to the vials containing the samples and standards and vortex mix for 2-3 seconds.
  18. Add 100 µL of the acetonitrile/butyl chloroformate (90/10, v/v) reagent to the vials containing the samples and standards and immediately vortex mix for 2-3 seconds after adding reagent to all vials.
  19. Allow the samples to sit at room temperature for approximately 5 minutes before evaporating to dryness using a TurboVap evaporator set at 40 °C and a nitrogen pressure of approximately 15 psi.
  20. Add 500 µL of acetonitrile/water (70/30, v/v) solution containing 0.1% H<sub>3</sub>PO<sub>4</sub> to each culture tube containing the samples and standards and vortex mix for 2-3 seconds.
  21. Transfer the samples along with the calibration standards to 1.5-mL autosampler vials and seal for analysis.
  22. Analyze the calibration standards and samples by HPLC with positive-ion electrospray tandem mass spectrometry.
  23. 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) 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 XDE-729 methyl and XDE-729 butyl at the 0.015 ng/mL calibration standard.

24. Re-analyze samples which contain concentrations of XDE-729 methyl and XDE-729 butyl ester are greater than 80% of the highest standard after diluting appropriately (max dilution 1:10) with acetonitrile/water (70/30, v/v) containing 0.1% H<sub>3</sub>PO<sub>4</sub> solution. If the dilution required is > 10X, a smaller aliquot of the sample extract should be selected at Step 11 and carry through the remainder of the procedure. The gross analyte concentration should be at least 30% above the lowest calibration standard and at least 20% less than the highest calibration standard.

#### Supplemental Notes

1. 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 used to prepare analytical standards, fortification solutions, and calibration standards.
2. The instrumental conditions may be modified to obtain optimal chromatographic separation and sensitivity.

Instrumental Conditions

*Typical HPLC Operating Conditions*

Instrumentation: Agilent 1290 Infinity LC System

Column: Acquity UPLC HSS T3  
 1.8  $\mu$ m, 50 x 2.1 mm

Column Temperature: 35 °C

Autosampler Temperature: 20 °C (Ambient)

Injection Volume: 10  $\mu$ L

Autosampler Wash: 30 seconds isopropanol/water (50/50, v/v)

Run Time: approximately 7 minutes

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

Mobile Phase Split: No

Equilibration Time: 0.5 minutes

Gradient:

Time (min)	Flow Rate (mL/min)	Mobile Phase A (percent)	Mobile Phase B (percent)
0.0	550	70	30
0.5	550	70	30
5.5	550	20	80
6.0	550	0	100
7.0	550	0	100

Flow Diverter:

Flow to Waste: 0.0 min → 2.5 min

Flow to Source: 2.5 min → 5.5 min

Flow to Waste: 5.5 min → end of run



*Typical Mass Spectrometry Operating Conditions*

Instrumentation: AB SCIEX QTRAP 5500 MS System  
 AB SCIEX Analyst version 1.6.2 data system

Ionization Mode: Electrospray

Polarity: Positive

Scan Type: MRM

Resolution: Q1 – unit, Q3 – unit

Curtain Gas (CUR): 30 psi

Collision Gas (CAD): Medium

Ion Source Gas 1 (GS1): 50 psi

Ion Source Gas 2 (GS2): 50 psi

Temperature (TEM): 600 °C

IonSpray Voltage (IS): 5500 volts

Entrance Potential (EP): 10 volts

Cell Exit Potential (CXP): 20 volts

Declustering Potential (DP): 80 volts

Dwell Time: 100 ms

<u>Analytes</u>	Precursor Ion, Q1 <i>m/z</i>	Product Ion, Q3 <i>m/z</i>	Collision Energy V
XDE-729 Methyl (345/250)	344.9	250.1	43
XDE-729 Methyl (345/235)	344.9	235.0	56
XDE-729 Methyl IS (351/256) (M+6)	350.8	256.1	43
XDE-729 Butyl (387/250)	387.0	250.1	43
XDE-729 Butyl (387/207)	387.0	207.1	69
XDE-729 Butyl IS (393/256) (M+6)	393.0	256.1	43