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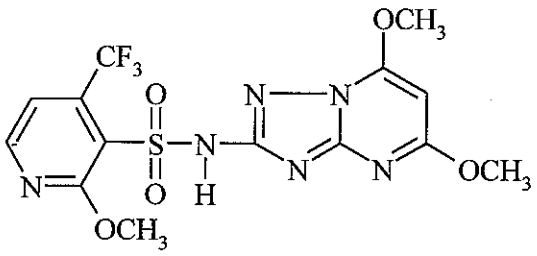
Determination of Residues of XDE-742 and its Metabolites in Soil and Sediment by Liquid Chromatography with Tandem Mass Spectrometry Detection

M. J. Hastings, A. E. Lindsey and G.E. Schelle

1. SCOPE

This method is applicable for the quantitative determination of residues of XDE-742 and its metabolites in soil and sediment. The method was validated for soil and sediment over the concentration range of 1.0-100 ng/g with a validated limit of quantitation of 1.0 ng/g.



XDE-742
CAS No. 422556-08-9

The molecular formula and nominal mass for the above structure and its metabolites are given in Table 1.

2. PRINCIPLE

Residues of XDE-742 and its metabolites are extracted from the soil by sonicating with 1 N hydrochloric acid solution, and shaking after the addition of methanol, to produce a methanol/1N hydrochloric acid solution (90:10). A mixed XDE-742 and metabolite stable isotope internal standard solution is added to the extraction solvent and an aliquot of the extract is evaporated to dryness. The sample is reconstituted in 0.1 N hydrochloric acid and purified using a polymeric 96-well solid phase extraction (SPE) plate. The SPE plate is washed with a water/methanol solution (75:25) and eluted with an acetonitrile/methanol solution (50:50). The eluate is evaporated to dryness and the residues are reconstituted in a water/methanol solution (90:10) containing 2 mM ammonium acetate. The purified extract is analyzed by high performance liquid chromatography with positive-ion electrospray (ESI) tandem mass spectrometry (LC/MS/MS).

3. SAFETY PRECAUTIONS

3.1. Each analyst must be acquainted with the potential hazards of the reagents, products, and solvents used in this method before commencing laboratory work. SOURCES OF INFORMATION INCLUDE MATERIAL SAFETY DATA SHEETS, LITERATURE, AND OTHER RELATED DATA. Safety information on non Dow AgroSciences LLC products should be obtained from the container label or from the supplier. Disposal of reagents, reactants, and solvents must be in compliance with local, state, and federal laws and regulations.

3.2. Acetonitrile and methanol are flammable and volatile and should be used in well-ventilated areas away from ignition sources.

3.3. Hydrochloric acid is corrosive and can cause severe burns. It is imperative that proper eye and personal protection equipment be used when handling all chemicals.

4. EQUIPMENT (Note 12.1.)

4.1. Laboratory Equipment

4.1.1. Balance, analytical, Model AE100, Mettler-Toledo, Inc., Columbus, OH 43240 .

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

4.1.3. Centrifuge, with rotor to accommodate 40-mLvials, Model Centra-GP8, Thermo International Equipment Company, Needham Heights, MA 02194.

4.1.4. Desiccator, glass, 250-mm I.D, catalog number 08-595-E, Fisher Scientific

4.1.5. Evaporator, TurboVap LV, Caliper Life Sciences, Hopkinton, MA 01748.

4.1.6. Hammer mill, with 1/8-inch or 3/16-inch screen, Model 2001, AGVISE Laboratories, Inc., Northwood, ND 58267.

4.1.7. Oven, Model OV-490A-2, Blue M Electric Company, Blue Island, IL 60406.

4.1.8. Pipettor, adjustable, Eppendorf, 10-100 µL, catalog number 05-402-48, Brinkmann Instruments, Inc., Westbury, NY 11590.

4.1.9. Pipettor, positive displacement, Gilson Microman M1000, 100-1000 µL, catalog number F148506, Gilson Inc, Middleton, WI 53562.

4.1.10. Shaker, variable speed reciprocating with box carrier, Model 6000, Eberbach Corporation, Ann Arbor, MI 48106.

4.1.11. Ultrasonic cleaner, Model 1200, Branson Cleaning Equipment Company, Shelton, CT 06484.

4.1.12. Vacuum manifold, 96-well, catalog number 121-9601, International Sorbent Technology Ltd, Hengoed, Mid Glamorgan UK and distributed by Biotage, Foxboro, MA 02035.

4.1.13. Vortex mixer, Model G-560, Scientific Industries, Inc., Bohemia, NY 11716.

4.2. Chromatographic System (Note 12.1.)

4.2.1. Column, analytical, Synergy Hydro RP, 2.0 x 50 mm, 4.0-µm, catalog number 008-4252-B0, Phenomenex, Torrance, CA 90501.

4.2.2. Liquid chromatograph, Symbiosis Pharma, Spark Holland Inc., Plainsboro, NJ 08536.

4.2.3. Mass spectrometer, Model API 4000, MDS/Sciex, Foster City, CA 94404.

4.2.4. Mass spectrometer data system, Analyst 1.4, MDS/Sciex.

5. GLASSWARE AND MATERIALS (Note 12.1.)

5.1. Bottle, media, 1000-mL, catalog number 02-543-03, Fisher Scientific, Pittsburgh, PA 15275.

5.2. Bottle, media, 2000-mL, catalog number 02-543-04, Fisher Scientific.

5.3. Collection plate, 2-mL, catalog number 121-5203, Biotage.

5.4. Cylinder, graduated mixing, 250-mL, catalog number 08-565E, Fisher Scientific.

5.5. Cylinder, graduated mixing, 500-mL, catalog number 08-565F, Fisher Scientific.

5.6. Cylinder, graduated mixing, 1000-mL, catalog number 08-564-5E Fisher Scientific.

5.7. Cylinder, graduated mixing, 2000-mL, catalog number 08-566-11H Fisher Scientific.

5.8. Dish, 42-mL aluminum weighing, catalog number 08-732, Fisher Scientific.

5.9. Flask, volumetric, 100-mL, catalog number 10-206D, Fisher Scientific.

5.10. Pipet, 3.2-mL disposable transfer, catalog number 13-711-7, Fisher Scientific.

5.11. Pipet, volumetric, 0.5-mL, catalog number 261-6010, National Scientific Company, Lawrenceville, GA 30243.

5.12. Pipet, volumetric, 1.0-mL, catalog number 261-6011, National Scientific Company.

5.13. Pipet, volumetric, 2.0-mL, catalog number 261-6012, National Scientific Company.

5.14. Pipet, volumetric, 3.0-mL, catalog number 261-6013, National Scientific Company.

5.15. Pipet, volumetric, 5.0-mL, catalog number 261-6015, National Scientific Company.

5.16. Pipet, volumetric, 10.0-mL, catalog number 261-6020, National Scientific Company.

5.17. Pipet, volumetric, 50.0-mL, catalog number 261-6060, National Scientific Company.

5.18. Pipetter, 10-mL bottle-top dispenser, catalog number 13-688-133, Fisher Scientific.

5.19. Pipetter, 50-mL bottle-top dispenser, catalog number 13-688-135, Fisher Scientific.

5.20. Pipetter, Brinkmann Eppendorf Repeater, catalog number 21-380-8, Fisher Scientific.

5.21. Pipetter tips, Brinkmann Eppendorf, 1-200-µL tip, catalog number 22351371, Brinkmann Instruments, Inc.

5.22. Repeater pipetter tips, 12.5-combitip tip, catalog number 21-380-8C, Fisher Scientific.

5.23. Repeater pipetter tips, 50-mL tip, catalog number 13-683-61G, Fisher Scientific.

5.24. SPE 96-well plate, Phenomenex Strata X, 33-µm, 30-mg packing, catalog number 8E-S100-TGB, Phenomenex.

5.25. Vial, autosampler, 2-mL, catalog number C4000-1, National Scientific Company.

5.26. Vial cap, for autosampler vial, catalog number C4000-54B, National Scientific Company.

5.27. Vial, 40-mL, with PTFE-lined screw cap, catalog number B7800-6, National Scientific Company.

6. REAGENTS, STANDARDS, AND PREPARED SOLUTIONS (Note 12.1.)

6.1. Reagents

6.1.2. Acetonitrile, Chrom AR HPLC grade, catalog number 2856, Mallinckrodt Baker, Inc., Paris, KY 40361.

6.1.3. Ammonium acetate, HPLC grade, catalog number A639-500, Fisher Scientific.

6.1.4. Adsorbent, Drierite, indicating, catalog number 07-578-4A, Fisher Scientific.

6.1.5. Dry ice, Continental Carbonic, Decatur, IL 62526.

6.1.6. Hydrochloric acid, 0.1 N, certified concentration, catalog number SA54-1, Fisher Scientific.

6.1.7. Hydrochloric acid, 1 N, certified concentration, catalog number SA48-1, Fisher Scientific.

6.1.8. Methanol, Chrom AR HPLC grade, catalog number 3041-10, Mallinckrodt Baker, Inc.

6.1.9. Nitrogen, refrigerated liquid, catalog number LQNI, BOC Gases, New Providence, NJ, 07974.

6.1.10. Water, HPLC grade, catalog number WX0004-1, EMD Chemicals, Gibbstown, NJ 08027.

6.2. Standards

6.2.1. Analytical standard information for XDE-742, 5-OH-XDE-742, 7-OH-XDE-742 and 6-Cl-7-OH-XDE-742 are listed in Table 1.

Compounds can be obtained from Test Substance Coordinator, Dow AgroSciences LLC, 9330 Zionsville Road, Building 304, Indianapolis, IN 46268-1054.

6.2.2 Stable isotope labeled internal standards information for XDE-742, 5-OH-XDE-742, 7-OH-XDE-742 and 6-Cl-7-OH-XDE-742 are listed in Table 1.

Obtain from Specialty Synthesis Group, Dow AgroSciences LLC, 9330 Zionsville Road, Building 306, Indianapolis, IN 46268-1054.

6.3. Prepared Solutions

6.3.1. acetonitrile/methanol (50:50)

Add 125 mL of acetonitrile to a 250-mL graduated mixing cylinder. Using a graduated cylinder, add 125 mL of methanol to the mixing cylinder containing acetonitrile. Place a glass stopper on the cylinder and invert several times to mix. Allow the solution to equilibrate to room temperature before use.

6.3.2. methanol containing 2 mM ammonium acetate

Weigh 0.15 grams of ammonium acetate into a tared 40-mL vial. Quantitatively transfer the ammonium acetate to a 1-L graduated mixing cylinder with methanol. Add methanol to the 1-L cylinder to bring the volume to the 1-L mark. Place a glass stopper on the cylinder and mix thoroughly.

6.3.3. water containing 2mM ammonium acetate

Weigh 0.15 grams of ammonium acetate into a tared 40-mL vial. Quantitatively transfer the ammonium acetate to a 1-L graduated mixing cylinder with water. Add water to the 1-L cylinder to bring the volume to the 1-L mark. Place a glass stopper on the cylinder and mix thoroughly.

6.3.4 water/methanol (75:25)

Add 150 mL of water to a 250-mL graduated mixing cylinder. Pipet 50 mL of methanol into the mixing cylinder containing water. Place a glass stopper on the cylinder and invert several times to mix. Allow the solution to equilibrate to room temperature prior to use.

6.3.5. water/methanol (90:10) containing 2 mM ammonium acetate

Weigh 0.31 grams of ammonium acetate into a tared 40-mL vial and quantitatively transfer to a 2-L bottle using two 50-mL aliquots of HPLC water. Add a further 1700-mL of water to the bottle using a 2-L graduated cylinder. Add 200 mL of methanol to the bottle using a 500-mL graduated cylinder. Allow the mixture to equilibrate to room temperature prior to use.

7. PREPARATION OF STANDARDS (Note 12.2)

7.1. Preparation of XDE-742 and Metabolite Spiking Solutions

7.1.1. Weigh 0.0100 g of XDE-742 analytical standard and quantitatively transfer to a 100-mL volumetric flask with methanol. Dilute to volume with methanol to obtain a 100.0-µg/mL stock solution.

7.1.2. Weigh 0.0100 g of the 7-OH-XDE-742 analytical standard and quantitatively transfer to a 100-mL volumetric flask with methanol. Dilute to volume with methanol to obtain a 100.0-µg/mL stock solution.

7.1.3. Weigh 0.0100 g of the 5-OH-XDE-742 analytical standard and quantitatively transfer to a 100-mL volumetric flask with methanol. Dilute to volume with methanol to obtain a 100.0-µg/mL stock solution.

7.1.4. Weigh 0.0100 g of the 6-Cl-7-OH-XDE-742 analytical standard and quantitatively transfer to a 100-mL volumetric flask with methanol. Dilute to volume with methanol to obtain a 100.0-µg/mL stock solution.

7.1.5. Pipet 10.0 mL of each of 100.0-µg/mL XDE-742 and metabolite stock solutions prepared in Sections 7.1.1-7.1.4. into a 100-mL volumetric flask and dilute to volume with methanol to obtain a 10.0-µg/mL mixed stock solution.

7.1.6. Pipet 10.0 mL of the 10.0-µg/mL mixed standard solution prepared in Section 7.1.5. into a 100-mL volumetric flask and adjust to volume with methanol to obtain a 1.0-µg/mL mixed spiking solution.

7.1.7. Pipet 5.0 mL of the 10.0-µg/mL mixed standard solution prepared in Section 7.1.5. into a 100-mL volumetric flask and adjust to volume with methanol to obtain a 0.5-µg/mL mixed spiking solution.

7.1.8. Pipet 10.0 mL of the 1.0-µg/mL mixed spiking solution prepared in Section 7.1.6. into a 100-mL volumetric flask and adjust to volume with methanol to obtain a 0.1-µg/mL mixed spiking solution.

7.1.9. Pipet 3.0 mL of the 1.0-µg/mL mixed spiking solution prepared in Section 7.1.6. into a 100-mL volumetric flask and adjust to volume with methanol to obtain a 0.03-µg/mL mixed spiking solution.

7.1.10. Pipet 10.0 mL of the 0.1-µg/mL mixed spiking solution prepared in Section 7.1.8. into a 100-mL volumetric flask and adjust to volume with methanol to obtain a 0.01-µg/mL mixed spiking solution.

7.2. Preparation of XDE-742 and Metabolite Stable Isotope Internal Standard Solutions

7.2.1. Weigh 0.010 g of the XDE-742 stable isotope analytical standard and quantitatively transfer to a 100-mL volumetric flask with methanol. Dilute to volume with methanol to obtain a 100.0-µg/mL stock solution.

7.2.2. Weigh 0.010 g of the 7-OH-XDE-742 stable isotope analytical standard and quantitatively transfer to a 100-mL volumetric flask with methanol. Dilute to volume with methanol to obtain a 100.0-µg/mL stock solution.

7.2.3. Weigh 0.010 g of the 5-OH-XDE-742 stable isotope analytical standard and quantitatively transfer to a 100-mL volumetric flask with methanol. Dilute to volume with methanol to obtain a 100.0-µg/mL stock solution.

7.2.4. Weigh 0.010 g of the 6-Cl-7-OH-XDE-742 stable isotope analytical standard and quantitatively transfer to a 100-mL volumetric flask with methanol. Dilute to volume with methanol to obtain a 100.0-µg/mL stock solution.

7.2.5. Pipet 1.0 mL of each of the 100.0-µg/mL stable isotope stock solutions prepared in Sections 7.2.1. – 7.2.4. into a 100-mL volumetric flask and adjust to volume with a water/methanol solution (90:10) containing 2 mM ammonium acetate to obtain a 1.0-µg/mL mixed internal standard solution.

7.2.6. Pipet 10.0 mL of the 1.0-µg/mL mixed stable isotope internal standard solution prepared in Section 7.2.5. into a 100-mL volumetric flask and adjust to volume with a water/methanol solution (90:10) containing 2 mM ammonium acetate to obtain a 0.10 µg/mL mixed stable isotope internal standard solution.

7.3. Preparation of XDE-742 and Metabolite Mixed Calibration Standards

7.3.1. Pipet 1.0 mL of each of the 100.0-µg/mL XDE-742 and metabolite stock solutions prepared in Sections 7.1.1. – 7.1.4. into a 100-mL volumetric flask and adjust to volume with a water/methanol solution (90:10) containing 2 mM ammonium acetate to obtain a 1.0-µg/mL mixed calibration standard stock solution.

7.3.2. Pipet 10.0 mL of the 1.0-µg/mL mixed calibration solution prepared in Section 7.3.1. into a 100-mL volumetric flask and adjust to volume with a water/methanol solution (90:10) containing 2 mM ammonium acetate to obtain a 0.1-µg/mL calibration solution.

7.3.3. Pipet 10.0 mL of the 0.1-µg/mL calibration solution prepared in Section 7.3.2. into a 100-mL volumetric flask and adjust to volume with a water/methanol solution (90:10) containing 2 mM ammonium acetate to obtain a 0.01-µg/mL calibration solution.

7.3.4. Prepare calibration solutions by pipetting 5.0 mL of the 0.1-µg/mL mixed stable isotope internal standard solution (Section 7.3.2.) into each 100-mL volumetric flask and then diluting the calibration standard stock solutions prepared in Sections 7.3.1.-7.3.3. with a water/methanol solution (90:10) containing 2 mM ammonium acetate as described in the table below:

Original Standard Concentration	Aliquot of Original Standard	Final Solution Volume	Calib. Soln. Final Concentration	Equivalent Sample Concentration [a]
µg/mL	mL	mL	ng/mL	ng/g
1.0	5.0	100.0	50.0	100.0
1.0	3.5	100.0	35.0	70.0
1.0	2.5	100.0	25.0	50.0
0.1	10.0	100.0	10.0	20.0
0.1	5.0	100.0	5.0	10.0
0.1	2.5	100.0	2.5	5.0
0.01	5.0	100.0	0.5	1.0
0.01	1.5	100.0	0.15	0.3

[a] The equivalent sample concentration is based on extracting a 5-g soil or sediment sample.

8. INSTRUMENTAL CONDITIONS (Note 12.3)

8.1. Typical Liquid Chromatography Operation Conditions for Quantitation of Residues

Instrumentation:	Spark Holland Symbiosis Pharma MDS/Sciex API 4000 LC/MS/MS System MDS/Sciex Analyst 1.4 data system
Column:	Phenomenex Synergi 4µ Hydro RP 50 x 2.0 mm, 4.0-µm
Column Temperature:	35 °C
Injection Volume:	50 µL
Run Time:	9.0 minutes
Mobile Phase:	A –methanol with 2 mM ammonium acetate B –water with 2 mM ammonium acetate
Flow Rate:	500 µL/min

Flow Diverter Program: 0.0 – 2.0 min: flow diverted to waste
2.0 – 5.0 min: flow to source
5.0 – end of run: flow diverted to waste

Gradient:	Time, min	A, %	B, %
	0:00	10	90
	6:00	100	0
	6:15	10	90

Equilibration: 2:45

8.2. Typical Mass Spectrometry Operating Conditions (Note 12.3)

Interface:	TurboIonSpray
Polarity:	Positive
Scan Type:	MRM
Resolution:	Q1 – unit, Q3 – low
Curtain Gas (CUR):	40
Collision Gas (CAD):	4.0
Temperature (TEM):	500 °C
Ion Source Gas 1 (GS1):	45
Ion Source Gas 2 (GS2):	75
Period 1	
Pre-Acquisition Delay:	2.0 min
Time:	3.0 min
IonSpray Voltage (IS):	5500
Smart Settling:	Off
MR Pause:	5 ms

Compound:	Ion *m/z*			
	Q1	Q3	Time, ms	Collision Energy, v
XDE-742 Quant	435	195	75	35
XDE-742 Conf	435	82	75	70
XDE-742 IS	438	198	75	35
5-OH-XDE-742 Quant	421	181	75	31
5-OH-XDE-742 Conf	421	148	75	37
5-OH-XDE-742 IS	425	185	75	31
7-OH-XDE-742 Quant	421	181	75	31
7-OH-XDE-742 Conf	421	148	75	37
7-OH-XDE-742 IS	425	185	75	31
6-Cl-7-OH-XDE-742 Quant	455	215	75	31
6-Cl-7-OH-XDE-742 Conf	455	148	75	37
6-Cl-7-OH-XDE-742 IS	459	219	75	31

8.3. Mass Spectra

Typical full-scan and product-ion mass spectra of XDE-742, its metabolites and stable isotope internal standards are presented in Figures 1-8.

8.4. Typical Calibration Curve

Typical calibration curves for the determination of XDE-742 and its metabolites in soil and sediment are presented in Figures 9-16.

8.5. Typical Chromatograms

Typical chromatograms of a standard, a control sample, a 1.0-ng/g (LOQ) recovery sample, and a 10-ng/g (10x LOQ) recovery sample for the determination of XDE-742 and metabolites in soil are presented in Figures 17-24.

9. DETERMINATION OF RECOVERY OF XDE-742 AND ITS METABOLITES FROM SOIL AND SEDIMENT

9.1. Method Validation

Validate the analytical procedure given in Section 9.3 by analyzing the following with each sample set:

At least one reagent blank.
At least one unfortified control.
At least one control fortified at the proposed limit of detection.
At least two controls fortified at the limit of quantitation.
At least two controls fortified at a level exceeding the expected residue concentration in the samples.

9.2. Sample Preparation

Prepare the soil samples for analysis by freezing with liquid nitrogen or dry ice and then grinding or chopping using a hammer mill with a 1/8-inch or 3/16-inch screen size. No preparation is required for sediment samples. However, sediment samples should be thoroughly stirred prior to use. Prepared soil samples should be stored frozen prior to analysis and sediment samples should be stored refrigerated prior to analysis.

9.3. Sample Analysis

9.3.1. Weigh 5.0 ± 0.05 g of the soil or sediment sample into a series of 40-mL vials with a PTFE-lined caps.

9.3.2. For recovery samples, add 50-µL aliquots of the spiking solutions to obtain concentrations ranging from 0.3 ng/g to 100 ng/g.

9.3.3. Add 2.0 mL of 1N hydrochloric acid to the sample vial.

9.3.4. Cap the sample vial and sonicate the sample for approximately 15 minutes.

9.3.5. Remove the sample from the ultrasonic bath and add 18.0 mL of methanol to the sample vial.

9.3.6. Cap the sample and place on a reciprocating shaker set at approximately 180 excursions/minute for 1 hour.

9.3.7. Remove the sample from the shaker and centrifuge at approximately 1800 rpm for 5 minutes.

9.3.8. Decant the extract into a 40-mL vial.

9.3.9. Add an additional 2.0 mL of 1N hydrochloric acid to the vial containing the soil sample. Cap the vial and sonicate the sample for approximately 10 minutes.

9.3.10. Remove the sample from the ultrasonic bath and add 18.0 mL of methanol to the sample vial.

9.3.11. Cap the vial and vortex mix the sample for approximately 30 seconds to ensure that the soil plug has been reconstituted in the extraction solvent.

9.3.12. Place the sample on a reciprocating shaker at approximately 180 excursions/minute for 30 minutes.

9.3.13. Remove the sample from the shaker and centrifuge the sample at approximately 1800 rpm for 5 minutes.

9.3.14. Combine the extract with the original extract from Step 9.3.8 by decanting the extract into the 40-mL vial containing the first extract.

9.3.15. Add 50 µL of the 1.0 µg/mL mixed XDE-742 stable isotope internal standard solution to each sample vial containing the combined sample extracts.

9.3.16. Cap the sample vial and invert several times to mix the sample extract.

9.3.17. Using a disposable graduated glass pipet, remove a 3.0-mL aliquot of the extract and dispense into a new 40-mL vial.

9.3.18. Evaporate the extract to dryness using a TurboVap evaporator set at approximately 40°C and a nitrogen flow rate of approximately 15 psi. **(Note: Ensure that the sample is not on the evaporator for longer than 20 minutes. Remove the sample vial immediately after evaporation.)**

9.3.19. Reconstitute the residue in 1.5 mL of 0.1N hydrochloric acid.

9.3.20. Sonicate the sample for approximately 15 minutes and vortex mix for approximately 10 seconds to ensure that the residuum has been reconstituted in the hydrochloric acid.

9.3.21. Purify the sample using the following SPE procedure (Note 12.4):

a. Condition a Phenomenex Strata X 96-well SPE plate (30-mg) with 1.0 mL of methanol followed by 1.0 mL of 0.1N hydrochloric acid. Dry the plate under full vacuum (approximately 15 inches of Hg) for 5 seconds between solvents.

b. Transfer the sample solution from Step 9.3.19 to the SPE plate. Pull the sample through the plate at approximately 1 mL/minute, discarding the eluate.

c. Rinse the sample vial with 1 mL of 0.1 N hydrochloric acid and transfer to the SPE plate. Pull the sample through the plate at approximately 1 mL/minute, discarding the eluate. Dry the plate under full vacuum for 5 seconds after the sample has eluted.

d. Wash the SPE plate with 1.0 mL of a water/methanol solution (75:25 v/v), discarding the eluate. Dry the SPE plate under full vacuum for 5 minutes.

e. Elute the XDE-742 and metabolites from the plate at approximately 1 mL/minute with two 1.0-mL aliquots of an acetonitrile/methanol solution (50:50), collecting the eluate in a 2-mL 96-deep well collection rack.

9.3.22. Transfer the sample from the 96-deep well collection rack sample to a 40-mL vial.

9.3.23. Evaporate the sample to dryness with nitrogen using a TurboVap evaporator set at approximately 40°C with a nitrogen flow rate of approximately 15 psi. **(Note: the sample should evaporate in approximately 5 minutes. Do not leave the sample on the evaporator longer than is necessary to evaporate the eluate.)**

9.3.24. Reconstitute the sample in 750 µL of a water/methanol solution (90:10 v/v) containing 2 mM ammonium acetate.

9.3.25. Sonicate the samples for approximately 5 minutes and vortex mix the samples for approximately 5 seconds.

9.3.26. Transfer each sample to an autosampler vial and cap with a crimp-top cap.

9.3.27. Chromatograph the samples and standard using the conditions given in Section 8, injecting the calibration standards throughout the run.

9.3.28. For sample extracts which contain XDE-742 and metabolite concentrations > 50 ng/mL (equivalent to >100 ng/g), dilute with a methanol/water solution (90:10) containing 2 mM ammonium acetate. Determine the suitability of the chromatographic system using the following criteria:

a. Standard curve linearity: Determine that the correlation coefficient equals or exceeds 0.98 for the least squares equation which describes the detector response as a function of standard curve concentration.

b. Peak resolution: Determine visually that sufficient resolution has been achieved for the analyte relative to any background interferences.

c. Appearance of chromatograms: Visually determine that the chromatograms resemble those shown in Figures 17-24 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 the 0.5-ng/mL calibration standard (equivalent to 1.0 ng/g of XDE-742 and or metabolites in a soil or sediment sample).

10. CALCULATIONS

10.1. Determination of Isotopic Crossover

In this assay, the analyte and internal standard 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 and should be addressed for accurate determination of concentrations.

10.1.1. To determine the isotopic crossover for XDE-742 and its metabolites and their respective stable isotopes, inject appropriate mixed XDE-742 and metabolite standard and mixed XDE-742 and metabolite stable isotope standards. Determine the peak areas for the analyte and internal standard as indicated below. For example, to determine the contribution of the unlabeled XDE-742 analyte to the stable isotope labeled XDE-742 internal standard:

XDE-742	*m/z* Q1/Q3 435.1/195.2
XDE-742 IS	*m/z* Q1/Q3 438.1/198.2

To determine the contribution of the unlabeled XDE-742 to the labeled XDE-742 internal standard:

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

$$\text{Crossover Factor (analyte}\rightarrow\text{ISTD)} = \frac{\text{peak area at } m/z \text{ 438.1/198.2}}{\text{peak area at } m/z \text{ 435.1/195.2}}$$

In a similar manner, to determine the contribution of the labeled XDE-742 stable isotope to the unlabeled XDE-742:

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

$$\text{Crossover Factor (ISTD}\rightarrow\text{ analyte)} = \frac{\text{peak area at } m/z \text{ 435.1/195.1}}{\text{peak area at } m/z \text{ 438.1/198.2}}$$

During method development, 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 (13.1, 13.2).

10.2. Calculation of Standard Calibration Curve

10.2.1. Inject the series of calibration standards described in Section 7.3 using the conditions listed in Section 8 and determine the peak areas for XDE-742 and its metabolites and internal standards as indicated below:

XDE-742 Quant	*m/z* Q1/Q3 435/195
XDE-742 Conf	*m/z* Q1/Q3 435/82
XDE-742 IS	*m/z* Q1/Q3 438/198
5-OH-XDE-742 Quant	*m/z* Q1/Q3 421/181
5-OH-XDE-742 Conf	*m/z* Q1/Q3 421/148
5-OH-XDE-742 IS	*m/z* Q1/Q3 425/185
7-OH-XDE-742 Quant	*m/z* Q1/Q3 421/181
7-OH-XDE-742 Conf	*m/z* Q1/Q3 421/148
7-OH-XDE-742 IS	*m/z* Q1/Q3 425/185
6-Cl-7-OH-XDE-742 Quant	*m/z* Q1/Q3 455/215
6-Cl-7-OH-XDE-742 Conf	*m/z* Q1/Q3 455/148
6-Cl-7-OH-XDE-742 IS	*m/z* Q1/Q3 459/219

10.2.2. For each standard, calculate the quantitation ratio.

For example, using the data for XDE-742 from injection no. 3, Figure 9:

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

$$\text{Quantitation Ratio} = \frac{\text{analyte peak area at m/z 435.1/195.2}}{\text{ISTD peak area at m/z 438.10/198.2}}$$

$$\text{Quantitation Ratio} = \frac{923000}{8870000}$$

$$\text{Quantitation Ratio} = 0.104$$

10.2.3. Prepare a standard curve by plotting the concentration of the analytes on the abscissa (x-axis) and the respective quantitation ratio on the ordinate (y-axis), as shown in Figures 9-16. Using linear regression analysis (13.3.) with a 1/x weighting (13.4.), determine the equation for the curve with respect to the abscissa (Note 12.5).

For example, using the XDE-742 data from Figure 9:

$$X = \left(\frac{Y - \text{intercept}}{\text{slope}}\right)$$

$$\text{XDE - 742 conc. (ng/mL)} = \left(\frac{\text{XDE - 742 quantitation ratio} - \text{intercept}}{\text{slope}}\right)$$

$$\text{XDE - 742 conc. (ng/mL)} = \left(\frac{\text{XDE - 742 quantitation ratio} - (0.0263)}{0.1595}\right)$$

10.3. Calculation of Percent Recovery for XDE-742 and its Metabolites

10.3.1. Determine the gross concentration in each recovery sample by substituting the peak area obtained into the above equation and solving for the concentration.

For example, using the data for XDE-742 data from injection no.10, Figure 17:

$$\text{XDE - 742 conc. (ng/mL)} = \left(\frac{\text{XDE - 742 quantitation ratio} - (0.0263)}{0.1595}\right)$$

$$\text{XDE - 742 conc. (gross ng/mL)} = \left(\frac{0.0960 - (0.0263)}{0.1595}\right)$$

$$\text{XDE - 742 conc. (gross)} = 0.4371 \text{ ng/mL}$$

Convert the concentration of ng/mL of XDE-742 found in the final sample extract prepared for analysis to ng/g of XDE-742 in the original soil sample as follows:

$$\text{XDE - 742 conc. (gross ng/g)} = 0.4371 \text{ ng/mL} \times \frac{(\text{nominal } 40 \text{ mL}/3 \text{ mL}) \times 0.75 \text{ mL}}{5 \text{ g}}$$

$$\text{XDE - 742 conc. (gross)} = 0.874 \text{ ng/g}$$

10.3.2. Determine the net concentration in each recovery sample by subtracting the concentration found at the retention time of each analyte in the untreated control sample from that of the gross analyte concentration in the recovery sample. For example, using the data for XDE-742 from injection 6, Figure 17:

$$\text{XDE-742 conc. (net ng/g)} = \text{XDE-742 conc. (gross µg/g)} - \text{XDE-742 conc. (control µg/g)}$$

$$\text{XDE-742 conc. (net ng/g)} = 0.874 \text{ ng/g} - 0.000 \text{ ng/g}$$

$$\text{XDE-742 conc. (net)} = 0.874 \text{ ng/g}$$

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

$$\text{Recovery} = \frac{\text{conc. found}}{\text{conc. added}} \times 100\%$$

$$\text{Recovery} = \frac{0.874\ \text{ng/g}}{1.000\ \text{ng/g}} \times 100\%$$

$$\text{Recovery} = 87\%$$

10.4. Determination of XDE-742 and its Metabolites in Soil and Sediment Samples

10.4.1. Determine the gross concentration of XDE-742 and its metabolites in each soil or sediment sample by substituting the respective peak area into the equation for the calibration curve and calculating the uncorrected residue result as described in Section 10.3.1.

10.4.2. For those samples that require correction for the method procedural recovery, use the average recovery of all the recovery samples at or above the limit of quantitation, as described in Section 9.1, from a given sample set to correct for method efficiency. For example, continuing with the data from Figure 17 and the average recovery from Table 2 for the samples analyzed on 15-Jul-2005:

$$\text{XDE-742 conc. (corrected ng/g)} = \text{XDE-742 conc. (gross ng/g)} \times \left(\frac{100}{\text{Average \% Recovery}}\right)$$

$$\text{XDE-742 conc. (corrected ng/g)} = 0.874\ \text{ng/g} \times \frac{100}{96}$$

$$\text{XDE-742 conc. (corrected)} = 0.910\ \text{ng/g}$$

10.5. Determination of Soil Moisture

10.5.1. Accurately weigh a 10-g portion of soil into a tared aluminum weighing dish.

10.5.2. Place the sample in an oven at 110 °C and allow to dry for a minimum of 16 hours.

10.5.3. Remove the sample from the oven and place in a desiccator containing Drierite adsorbent. Re-weigh the sample when it has cooled to room temperature.

10.5.4. Calculate the percent moisture (dry weight basis) as follows:

$$\text{Percent Moisture (dry weight basis)} = \frac{\text{water, g}}{\text{dry soil, g}} \times 100$$

$$\text{Percent Moisture (dry weight basis)} = \frac{\left(\text{sample weight before drying, g}\right) - \left(\text{sample weight after drying, g}\right)}{\text{sample weight after drying, g}} \times 100$$

10.6. Determination of Dry Weight Concentrations of XDE-742 and Metabolites in Soil and Sediment

10.6.1. Determine the analyte concentrations in the sample as described in Section 10.4.

10.6.2. Determine the soil moisture as described in Section 10.5.

10.6.3. Determine the dry weight analyte concentrations in the samples as follows:

$$\text{XDE-742 conc. (dry weight ng/g)} = \text{XDE-742 conc. (ng/g)} \times \left(1 + \frac{\%\,\text{Moisture}}{100}\right)$$

11.2. Confirmation of Residue Identity

The presence of XDE-742 and its metabolites is confirmed by comparing the liquid chromatography retention times of the analyte in the calibration standards with those found in the samples while monitoring analyte specific MS/MS transitions. Further confirmation is achieved by the observation of a second structurally significant product-ion transition (Section 8) observed at the same retention time.

11.3. Assay Time and Stopping Points

A typical analytical run would consist of eight standards encompassing the expected range of sample concentrations, a reagent blank, a control (a non-fortified sample), a minimum of three fortified controls (two of which must be at the LOQ), and 15 samples. This typical analytical set can be prepared in approximately 6 hours followed by the chromatographic analysis.

XDE-742 is moderately unstable when stored for >24 hours in the extraction solvent; therefore no acceptable "stopping points" are suggested in this procedure before Step 9.3.18. Acceptable stopping points (stored in a refrigerator) after Step 9.3.18 are:

a. Step 9.3.21.
b. Step 9.3.23.
c. Step 9.2.25.

11.4. Standardization of Phenomenex Strata X SPE Elution Profile

Lot-to-lot variation in the Phenomenex Strata X SPE 96-well plate may influence the elution profile of XDE-742 and its metabolites. Obtain an elution profile for each new lot of SPE plates used to ensure optimum recovery and clean-up efficiency. The following procedure can be used:

11.4.1. Pipet 1.5 mL of 0.1 N hydrochloric acid into a 40-mL vial.

11.4.2. Transfer 50 µL of a 1.0-µg/mL mixed XDE-742 standard solution (Section 7.1.6.) to the culture tube containing the 0.1 N hydrochloric acid and vortex-mix the solution for approximately 5 seconds.

11.4.3. Place a Phenomenex Strata X SPE 96-well plate on a vacuum manifold. Determine the SPE elution profile using the standard solution prepared according to the following procedure:

a. Condition the Phenomenex Strata X 96-well SPE plate (30-mg) with 1.0 mL of methanol followed by 1.0 mL of 0.1N hydrochloric acid. Dry the plate under full vacuum (approximately 15 inches of Hg) for 5 seconds between solvents.

b. Transfer the solution from Step 11.4.2 to the SPE plate. Pull the solution through the plate at approximately 1 mL/minute, discarding the eluate.

c. Rinse the 40-mL vial with 1 mL of 0.1 N hydrochloric acid and transfer to the SPE plate. Pull the solution through the plate at approximately 1 mL/minute, discarding the eluate. Dry the plate under full vacuum for 5 seconds after the sample has eluted.

d. Wash the SPE plate with 1.0 mL of a water:methanol solution (75:25 v/v), discarding the eluate. Dry the SPE plate under full vacuum for 5 minutes.

e. Elute the XDE-742 and metabolites from the plate at approximately 1 mL/minute with six 0.5-mL aliquots of an acetonitrile:methanol solution (50:50), collecting the eluate in separate wells of a 2-mL 96-deep well collection rack..

11.4.4. Transfer the sample from the 96-deep well collection rack sample to a 40-mL vial.

11.4.5. Evaporate the sample to dryness with nitrogen using a TurboVap evaporator set at approximately 40°C with a nitrogen flow rate of approximately 15 psi. **(Note: the sample should evaporate in approximately 5 minutes. Do not leave the sample on the evaporator longer than is necessary to evaporate the eluate.)**

11.4.6. Reconstitute the sample in 750 µL of a water/methanol solution (90:10 v/v) containing 2 mM ammonium acetate.

11.4.7. Sonicate the samples for approximately 5 minutes and vortex mix the samples for approximately 5 seconds.

11.4.8. Transfer each sample to an autosampler vial and cap with a crimp-top cap.

11.4.9. Chromatograph the samples and standard using the conditions given in Section 8, injecting the calibration standards throughout the run.

11.4.10. Calculate the percent recovery using an external standard technique.

If the elution profile differs from that suggested in the method shown in Figure 25, adjust the volume of acetonitrile/methanol (50:50) to be used for elution in Step 9.3.21.e of the sample analysis procedure.

12. NOTES

12.1. 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 supplies are assumed to be readily available and are, therefore, not listed.

12.2. Section 7 provides suggested concentrations for standard preparation. Other dilution schemes may be followed.

12.3. Operating conditions may be modified to obtain optimal separation or sensitivity.

12.4. Before using each lot of Phenomenex Strata X 96-Well SPE Plates, determine the elution profile as described in Section 11.4.

12.5. A quadratic curve fit may also be used where appropriate.

13. REFERENCES

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Table 1. Identity and Structure of XDE-742, its Metabolites and Stable Isotope Internal Standards

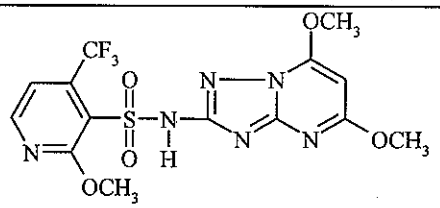
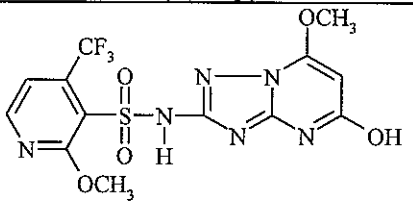
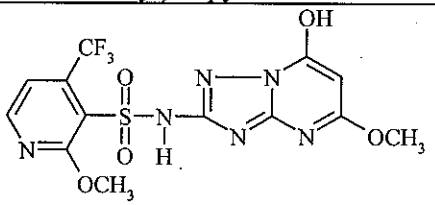
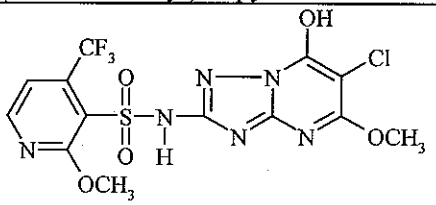
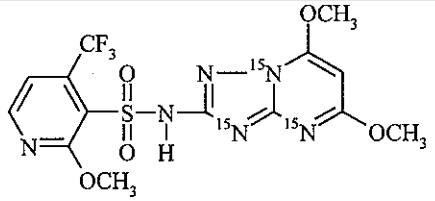
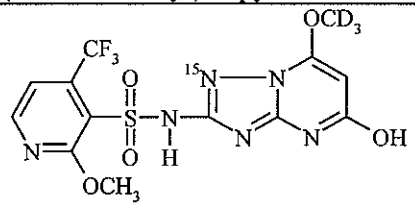
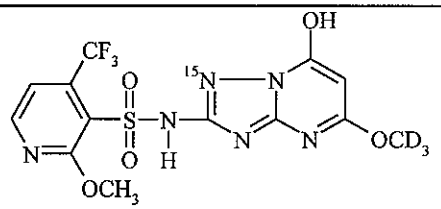
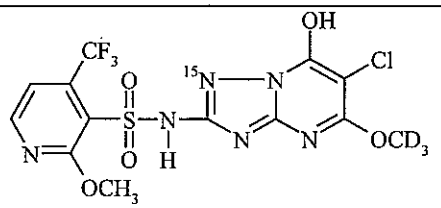
Common Name of Compound	Structure and CAS Name
XDE-742 Molecular Formula: $C_{14}H_{13}F_3N_6O_5S$ Nominal Mass: 434 CAS Number: 422556-08-9	 *N*-(5,7-dimethoxy[1,2,4]triazolo[1,5-a]pyrimidin-2-yl)-2-methoxy-4-(trifluoromethyl)-3-pyridinesulfonamide
5-OH-XDE-742 Molecular Formula: $C_{13}H_{11}F_3N_6O_5S$ Nominal Mass: 420 CAS Number: N/A	 *N*-(5-hydroxy-7-methoxy[1,2,4]triazolo[1,5-*a*]pyrimidin-2-yl)-2-methoxy-4-(trifluoromethyl)-3-pyridinesulfonamide
7-OH-XDE-742 Molecular Formula: $C_{13}H_{11}F_3N_6O_5S$ Nominal Mass: 420 CAS Number: N/A	 *N*-(7-hydroxy-5-methoxy[1,2,4]triazolo[1,5-*a*]pyrimidin-2-yl)-2-methoxy-4-(trifluoromethyl)-3-pyridinesulfonamide
6-Cl-7-OH-XDE-742 Molecular Formula: $C_{13}H_{10}ClF_3N_6O_5S$ Nominal Mass: 454 CAS Number: N/A	 *N*-(6-chloro-7-hydroxy-5-methoxy[1,2,4]triazolo[1,5-a]pyrimidin-2-yl)-2-methoxy-4-(trifluoromethyl)pyridine-3-sulfonamide

Table 1. (Cont.) Identity and Structure of XDE-742, its Metabolites and Stable Isotope Internal Standards

Common Name of Compound	Structure and CAS Name
XDE-742 IS Molecular Formula: $C_{14}H_{13}F_3N_3{}^{15}N_3O_5S$ Nominal Mass: 437 CAS Number: N/A	 *N*-(5,7-dimethoxy[^{15}N-1,2,4]triazolo[1,5-a]pyrimidin-2-yl)-2-methoxy-4-(trifluoromethyl)-3-pyridinesulfonamide
5-OH-XDE-742 IS Molecular Formula: $C_{13}H_8D_3F_3N_5{}^{15}N\,O_5S$ Nominal Mass: 424 CAS Number: N/A	 *N*-(5-hydroxy-7-methoxy[^{15}N-1,2,4]triazolo[1,5-a]pyrimidin-2-yl)-2-methoxy-4-(trifluoromethyl)pyridine-3-sulfonamide-d3
7-OH-XDE-742 IS Molecular Formula: $C_{13}H_8D_3F_3N_5{}^{15}NO_5S$ Nominal Mass: 424 CAS Number: N/A	 *N*-(7-hydroxy-5-methoxy[^{15}N-1,2,4]triazolo[1,5-a]pyrimidin-2-yl)-2-methoxy-4-(trifluoromethyl)pyridine-3-sulfonamide-d3
6-Cl-7-OH-XDE-742 IS Molecular Formula: $C_{13}H_7D_3ClF_3N_5{}^{15}NO_5S$ Nominal Mass: 458 CAS Number: N/A	 *N*-(6-chloro-7-hydroxy-5-methoxy[^{15}N-1,2,4]triazolo[1,5-a]pyrimidin-2-yl)-2-methoxy-4-(trifluoromethyl)pyridine-3-sulfonamide-d3