Method Validation Study for the Determination of Residues of XDE-729 and its Metabolites in Surface Water, Ground Water and Drinking Water by Liquid Chromatography with Tandem Mass Spectrometry

ABSTRACT

A study was conducted to provide validation data for the determination of residues of XDE-729 and its metabolites in water as well as to support the stated limit of quantitation (LOQ) established at 0.05 µg/L per analyte in water.
For the validation, drinking (tap), surface (river) and ground (well) water were chosen. Untreated control samples were fortified at 0.05 µg/L, and at 0.5 µg/L with analytes.

Method Principle
10.0 mL of water sample (surface water, ground water and drinking water) are transferred into 15 mL centrifuges tubes. For reagent blank and untreated samples, 10.0 mL aliquot of ultra-pure water aliquot is added. To all samples, 1.0 mL of 10% formic acid solution is added. The solution is then purified using a Strata-X reversed-phase solid-phase extraction (SPE) column. After elution from the SPE column with methanol, the eluate is dried and reconstituted with an acetonitrile:water:formic acid (10:90:0.1, v/v/v) solution. The sample is analyzed by liquid chromatography with positive-ion electrospray ionization tandem mass spectrometry (LC-MS/MS).
Average recoveries at each fortification level ranged between 70-110 %. The relative standard deviation (RSD) did not exceed the level of ± 20% at any fortification level for either analyte, and interferences were negligible.
This method validation was conducted to satisfy the requirements of the European Council Directive 91/414/EEC, as amended by European Commission Directive 96/46/EC, and the European Commission Guidance Documents on Residue Analytical Methods, SANCO/825/00 rev. 8.1.

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

INTRODUCTION

An analytical procedure for the determination of residues of XDE-729 and its metabolites in waters by Liquid Chromatography with Tandem Mass Spectrometry Detection was developed at Dow AgroSciences LLC.

In the present study the method was revised to improve the recoveries and found to be suitable for the determination of residues of XDE-729 and its metabolites in waters over the concentration range of 0.05-0.5 µg/L. The validated limit of quantitation of the method was 0.05 µg/L.

ANALYTICAL

Preparation and Storage of Samples

The method validation was carried out on three water specimens: surface water, ground water and drinking water. The drinking water was obtained from a “drinking water” tap at the Mogi Mirim Regulatory Laboratory Test Facility, the ground water was obtained from a well in the Dow AgroSciences Field Station in Mogi-Mirim City, São Paulo State, Brazil and the surface water was obtained from the River Sapezal, in Mogi-Mirim City, São Paulo State, Brazil. All samples were collected in the same day, September 29th of 2011.

Specimen	Sample number
Ground Water	110718-001-0001
Surface Water	110718-001-0002
Drinking Water	110718-001-0003

On receipt the specimens were stored at approximately -20 °C before and after analysis.

Characterisation of Samples

The water specimens were characterised at NL Laboratórios, in Mogi-Mirim City, São Paulo State, Brazil. The laboratory is capable of following Brazilian laws for water analysis, such as stated by the Ministry of Environmental. As this is not a GLP facility, the data generated are non-GLP. Details of the characterisation results are as follows:

Sample Number	pH	Turbidity NTU	Total Hardness mg/L as $CaCO_3$	Total Organic Carbon (mg/L)	Chemical Oxygen Demand (mg/L)	Total Suspended Solids (mg/L)
110718-001-0001	7.22	0.23	68.1	<2.0	<2.0	<2.0
110718-001-0002	6.46	4.74	4.0	<1.0	13.1	<2.0
110718-001-0003	7.29	0.34	68.1	<2.0	<2.0	<2.0

Certified copies of the Certificates of Analysis of these specimens are included in the raw data package.

Laboratory Equipment

Balance, analytical, Model XP 205DR, Mettler-Toledo, Inc.
Balance, pan, Model XS802S, Mettler-Toledo, Inc.
Evaporator, TurboVap Concentration Workstation, Zymark/Biotage
Grinder, Model UM 25, Stephan, Inc.
Pipetter, adjustable electronic, model Research Pro, 5-100 µL, 50-1000 µL, 100-5000 µL, Eppendorf GmbH.
Ultrasonic cleaner, Model 5510, Branson Cleaning Equipment Company.
Vortex mixer, Model Genie 2, Fisher Scientific, Inc.
Graduated cylinders with cap, 50, 100, 500, 1000 and 2000-mL, Corning Brasil Vidros Especiais Ltda.
Flask, volumetric, 50 and 100-mL, Corning Brasil - Vidros Especiais Ltda.
Pipette tips, 200-µL, 1000-µL and 5000-µL capacity, Eppendorf GmbH.
Water purifier, Barnstead EASYpureII, Barnstead/Thermolyne Corp.

Chromatographic Equipment

Column, analytical, Zorbax SB-C8, 4.6 x 75 mm, 3.5-µm, catalog number 866953-906, Agilent Technologies.

Phenomenex Strata X 33 µm, 60-mg packing, catalog number 8E-Sl00-TGB, Phenomenex.
Vial, autosampler, 2-mL, Agilent Technologies.
Vial cap, for autosampler vial, Agilent Technologies.
Liquid chromatograph autosampler, Model 1100, Agilent Technologies.
Liquid chromatograph quaternary pump, Model 1100, Agilent Technologies.
Liquid chromatograph degasser, Model 1100, Agilent Technologies.
Mass spectrometer, Model API 4000, Applied Biosystems.
Mass spectrometer data system, Analyst 1.5, Applied Biosystems.

Reagents, Chemicals and Miscellaneous

Acetonitrile, methanol, all HPLC grade (JT Baker or equivalent).
Ultra pure water (supply at Mogi Mirim Regulatory laboratory).
Formic acid (100 %, Merck).

Preparation of Solutions

Water + 0.1% Formic acid (100:0.1, v/v) (Mobile phase B, SPE cleanup)
Measure 1000-mL of deionized water using a 1000-mL graduated cylinder and transfer to 1-L bottle. Pipet 1.0 mL of formic acid into the same flask. Cap the bottle and shake to mix well.

Methanol + 0.1% Formic acid (100:0.1, v/v) (Mobile phase A)
Measure 1000-mL of Methanol HPLC grade using a 1000-mL graduated cylinder and transfer to 1-L bottle. Pipet 1.0 mL of formic acid into the same flask. Cap the bottle and shake to mix well.

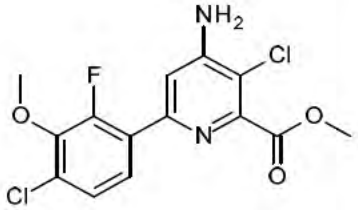
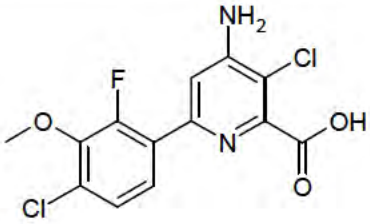
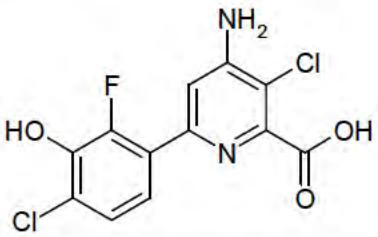
Acetonitrile/water/formic acid (10:90:0.1, v/v) (SPE reconstitution solvent)
Measure 100 mL of acetonitrile using a 250-mL graduated cylinder and transfer to a l-L bottle. Next measure 900 mL of deionized water using a 1000-mL graduated cylinder and transfer to the same bottle. Pipet 1.0 mL of formic acid into the media bottle. Cap the bottle and shake to mix well. Allow the solution to equilibrate to room temperature before use.

Water/formic acid (90:10, v/v) (acidification solution, preparation)

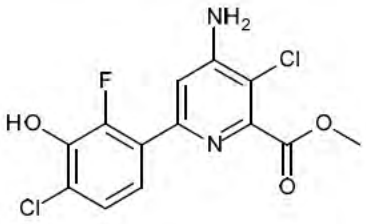
Measure 900-mL of deionized water using a 1000-mL graduated cylinder and transfer to 1-L bottle. Measure 100 mL of formic acid using a 250-mL graduated cylinder and transfer to the same bottle. Stopper the bottle and shake to mix well.

Standards

Following references substances were obtained from the Test Substance Coordinator, Dow AgroSciences LLC.

	Reference Substances	Structure
Common Name	XDE-729 methyl	
Synonyms	XDE-729 ME, XDE-729 methyl ester, XR-729 methyl, X11393728	
IUPAC Nomenclature	methyl 4-amino-3-chloro-6-(4-chloro-2-fluoro-3-methoxyphenyl)pyridine-2-carboxylate	
CAS Number	943831-98-9	
Molecular Weight	345.16 g/mol	
Lot number	XW7-38246-49	
Inventory #	TSN031117-0002 / AS 427	
Purity	97.4%	
Common Name	X11393729	
Synonyms	X'729, XR-729 acid, XDE-729 acid	
IUPAC Nomenclature	4-amino-3-chloro-6-(4-chloro-2-fluoro-3-methoxy-phenyl)-pyridine-2-carboxylic acid	
CAS Number	943832-60-8	
Molecular Weight	331.13 g/mol	
Lot number	DC6-E2622-77	
Inventory #	TSN030751-0004 / AS 429	
Purity	99.0%	
Common Name	X11449757	
Synonyms	X'757, Des-methyl-XR-729	
IUPAC Nomenclature	4-amino-3-chloro-6-(4-chloro-2-fluoro-3-hydroxyphenyl)-picolinic acid	
Molecular Weight	317.11 g/mol	
Lot number	YB1-100780-103	
Inventory #	TSN031413-0003 / AS 428	
Purity	99.0%	

Common Name	X11406790
Synonyms	X'790
CAS Nomenclature	methyl 4-amino-3-chloro-6-(4-chloro-2-fluoro-3-hydroxyphenyl)pyridine-2-carboxylate
Molecular Weight	331.13 g/mol
Lot number	DE3-037988-95
Inventory #	TSN031109-0001 / AS 426
Purity	99.0%



In addition, the following reagents were be used as internal standards:

Internal Standard	TSN Number	Lot Number	Percent Purity	Recertification Date	Reference
X11278577[a]	301413	DC6-112253-40	99.0	20-Nov-2016	FA&PC11-000133
X12278779[b]	301414	DC6-112253-42	100.0	12-Nov-2013	FA&PC11-000134
X12281837[c]	301486	DC6-112253-45	99.0	22-Nov-2016	FA&PC11-000147
X12280425[d]	301467	DC6-112253-43	100.0	20-Nov-2016	FA&PC11-000146

[a] Synonyms: XDE-729 methyl M+6, XDE-728 M+6
[b] Synonyms: XDE-729 acid M+6, XDE-729 M+6
[c] Synonyms: X11449757 M+6
[d] Synonyms: X11406790 M+6

EXPERIMENTAL

Instrumental Conditions

Typical HPLC Operating Conditions
(Supplemental Note 1)

Instrumentation:	Agilent Model 1100 autosampler Agilent Model 1100 quaternary pump Agilent Model 1100 degasser MDS/Sciex API 4000 LC/MS/MS System MDS/Sciex Analyst 1.5 data system
Column:	Zorbax SB-C8 4.6 x 75 mm, 3.5-µm
Column Temperature:	25 °C
Injection Volume:	25 µL
Run Time:	12.0 minutes

Mobile Phase: A – methanol + 0.1% formic acid
B – water + 0.1% formic acid

Flow Rate: 800 µL/min

Gradient:

Time, min	Solvent A, %	Solvent B, %
0.0	10	90
7.0	100	0
9.0	100	0
9.1	10	90
12.0	10	90

Flow Diverter Program: 1) 0.0 to 3.5 min: flow to waste
2) 3.5 to 9.0 min: flow to source
3) 9.0 to 12.0 min: flow to waste

Typical Mass Spectrometry Operating Conditions

Interface:	Electrospray
Polarity:	Positive
Scan Type:	MRM
Resolution:	Q1 – unit, Q3 – unit
Curtain Gas (CUR):	20
Collision Gas (CAD):	6
Temperature (TEM):	550 °C
Ion Source Gas 1 (GS1):	55
Ion Source Gas 2 (GS2):	40

Period 1

Pre-acquisition Delay:	0.0 min
Acquisition Time	10.0 min
IonSpray Voltage (IS):	5500 volts
Entrance Potential (EP):	10 volts

Transitions:	Q1 Ions	Q3 Ions	Time (ms)	CE/CXP	DP
XDE-729 methyl (345/285)	345.0	285.1	200	31/8	71
XDE-729 methyl (345/250)	345.0	250.1	200	45/14	71
XDE-729 acid (331/250)	330.8	250.0	200	31/16	80
XDE-729 acid (331/235)	330.8	235.0	200	43/16	80
X'790 (331/236)	331.0	236.0	200	33/14	80
X'790 (331/271)	331.0	271.0	200	45/3	80
X'757 (317/236)	317.0	236.0	200	31/14	80
X'757 (317/271)	317.0	270.9	200	31/14	80
XDE-729 methyl M+6	351.0	256.0	200	41/35	80
XDE-729 acid M+6	336.8	256.0	200	41/35	60
X'790 M+6	337.0	242.0	200	49/32	80
X'757 M+6	323.0	242.0	200	45/18	80

Representative spectra and calibration curve shown in Figures 1-4 and 53-60. Typical chromatograms for the determination of XDE-729 and its metabolites are illustrated in Figures 5-52.

Preparation of Standard Solutions

Preparation of XDE-729 methyl, XDE-729 acid, X11406790 and X11406757 Stock Solutions (Supplemental Note 2)

Weigh out 100.0 mg of XDE-729 methyl and XDE-729 acid reference standards. Quantitatively transfer each analyte into a separate 100 mL volumetric flask with methanol. Dilute to volume to obtain stock solutions of 1000 µg/mL.

Weigh out 25.0 mg of X11406790 reference standard. Quantitatively transfer the analyte into 25 mL volumetric flask with methanol. Dilute to volume to obtain stock solution of 1000 µg/mL.

Weigh out 2.0 mg of X11406757 reference standard. Quantitatively transfer the analyte into 100 mL volumetric flask with methanol. Dilute to volume to obtain stock solution of 20 µg/mL. (Supplemental Note 3)

Using volumetric pipettes, transfer 10 mL of each 1000 µg/mL solutions of XDE-729 methyl, XDE-729 acid and X11406790 into the same 100 mL volumetric flask. Dilute to volume with methanol to obtain a 100 µg/mL mixed spiking solution containing the three analytes.

Using volumetric pipettes, transfer 10 mL of the mixed spiking solution prepared above and 50 mL of the spiking solution of X11406757 (containing 20 µg/mL of the analyte) into the same 100 mL volumetric flask. Dilute to volume with methanol to obtain a 10 µg/mL mixed spiking solution containing the four analytes.

Prepare additional mixed spiking solutions by further diluting the 10 µg/mL mixed spiking solution containing the four analytes with methanol as follows. These spiking solutions are used to prepare the mixed calibration standards:

Mixed Spiking Solution Conc.	Volume of Mixed Spiking Solution	Volume of Final Spiking Solution	Final Spiking Solution Conc.
µg/mL	mL	mL	µg/mL
10.0	10.0	100	1.0
(1.0) [a]	5.0	100	0.05
(0.05) [a]	1.5	100	0.015

[a] The numbers in parenthesis indicates the solution for the series dilution.

Preparation of Spiking Solutions for Fortification

Prepare mixed spiking solutions for fortification by further diluting the mixed spiking solutions of XDE-729 methyl, XDE-729 acid, X11406790 and X11406757 using methanol as follows.

Mixed Spiking Solution Conc.	Volume of Mixed Spiking Solution	Volume of Final Spiking Solution	Final Spiking Solution Conc.	Equivalent Sample Conc. [a]
µg/mL	mL	mL	ng/mL	µg/L
1.0	5.0	50	100	1.0
0.05	5.0	50	5	0.05
0.015	5.0	50	1.5	0.015

[a] The equivalent samples concentration is based on fortifying 10.0 mL water sample with 100 µL of spiking solution.

Preparation of Stock Solutions and Spiking Solutions of Internal Standards

Weigh out 5.0 mg of XDE-729 methyl M+6, XDE-729 acid M+6 and X11406790 M+6 labeled reference standards. Quantitatively transfer each of the three internal standards into separated 10 mL volumetric flasks with methanol. Dilute to volume to obtain stock solutions of 500 µg/mL.

Weigh out 2.0 mg of X11449757 M+6 stable-isotope labeled reference standard. Quantitatively transfer into a 100 mL volumetric flask with methanol. Dilute to volume to obtain a stock solution of 20 µg/mL. (Supplemental Note 3)

Using positive displacement pipette, transfer 500 µL of each 500 µg/mL solutions of XDE-729 methyl M+6, XDE-729 acid M+6 and X11406790 M+6 into the same 50 mL volumetric flask. Dilute to volume with methanol to obtain a 5 µg/mL mixed spiking solution containing the three internal standards.

Using positive displacement pipette, transfer 5.0 mL of the above 5.0 µg/mL mixed internal standard solution and 1.25 mL of the 20 µg/mL spiking solution of X11449757 M+6 stable-isotope into the same 50 mL volumetric flask. Dilute to volume with methanol to obtain a 0.50

µg/mL mixed internal standard spiking. This spiking is used to prepare the mixed calibration standards.

Using a volumetric pipette, transfer 10 mL of the above 0.50 µg/mL mixed internal standard solution into a 100 mL volumetric flask. Dilute to volume with methanol to obtain a 50 ng/mL mixed internal standard spiking. This spiking is used in the sample preparation.

Preparation of Calibration Standards

Prepare calibration standards by using the spiking solutions described above as shown in the following table. Use a acetonitrile/water/formic acid (10/90/0.1, v/v/v) solution to dilute the standards. Add 100 µL of 0.5 µg/mL mixed internal standard spiking solution to each standard. The final concentration of the individual internal standard in each calibration standard is 5.0 ng/mL.

Spiking Solution Conc.	Volume of Spiking Solution	Final Volume of Cal. Std.	Final Cal. Std. Conc. of Analyte	Equivalent Sample Conc.[a]
µg/mL	µL	mL	ng/mL	µg/L
1.0	500	25.0	20	2.0
1.0	250	25.0	10	1.0
1.0	125	25.0	5.0	0.5
0.05	1000	25.0	2.0	0.2
0.05	500	25.0	1.0	0.1
0.05	250	25.0	0.50	0.05
0.015	250	25.0	0.15	0.015

[a] The equivalent samples concentration is based on a diluting factor of 10.

Method Validation

The accuracy and the precision of the proposed residue method were determined using freshly fortified untreated control water samples. Accuracy was calculated as the percent recovery while the precision was calculated using statistical treatments such as average and relative standard deviation (RSD).

The ruggedness of the method was demonstrated by conducting the validation on more than one day. Recovery samples were prepared on the day of analysis by freshly fortifying the untreated control water samples with the appropriate amounts of each analyte to result in the number of replicates indicated below. Fortification solutions were applied directly to the matrix using a volume delivery device with appropriate accuracy.

Analysis Procedure

For reagent blank, transfer 10.0 mL of ultra-pure water into a 15 mL centrifuge tube.

For control samples, transfer 10.0 mL of water sample (surface water, ground water and drinking water) into a 15 mL centrifuge tube.

For fortified samples, transfer 10.0 mL of control water sample into a 15 mL centrifuge tube. Add the appropriate volume of the spiking solution to obtain fortified samples at LOD, LOQ and a higher concentration level (0.015, 0.05 and 0.50 µg/L, respectively). Vortex a few second to mix well.

Add 1.0 mL of 10% formic acid to all the samples.

Solid-Phase Extraction

Purify the sample with the following SPE procedure:

a. Place a Strata-X reversed-phase SPE column (3 mL/60 mg) on the vacuum manifold.
b. Condition the SPE column with 3.0 mL of methanol followed by 3.0 mL of water/formic acid (100/0.1, v/v). Dry the SPE column under full vacuum for 5 seconds after each condition step.
c. Transfer the entire sample volume to the SPE column. Draw the sample through the column at a flow rate of approximately 1.0 mL/min. Discard the flow through.
d. Rinse the sample vial with 1.0 mL of water/formic acid (100/0.1, v/v) and load into the column. Draw the solvent through the column at a flow rate of approximately 1.0 mL/min. Discard the flow through. Dry the SPE column under full vacuum for 5 seconds after the rinse step.
e. Elute the analytes from the SPE column with 3x 500 µL aliquots of methanol. Collect the eluate in clean 15 mL centrifuge tubes.

Further Sample Preparation and MS analysis

Add 100 µL of 50 ng/mL mixed internal standard to the eluate. Evaporate the sample to near dryness using a TurboVap evaporator with the water bath temperature set at 40 °C and the pressure at approximately 7 psi.

Reconstitute sample with 1.0 mL of acetonitrile/water/formic acid (10/90/0.1, v/v/v). Vortex the sample for a few seconds, then sonicate for 2-3 minutes. Vortex a few seconds again to mix well.

Transfer approximately 1.0 mL of the sample into the 2.0 mL glass autosampler vial for the LC/MS/MS analysis.

Calculations

A series of calibration standards were prepared as described in the standard preparation section, analyzed using the conditions listed in the instrument section and determined the peak areas for thes analytes. As example, is given XDE-729 methyl:

XDE-729 methyl Quantitation ion	Q1/Q3 345.0/285.1
XDE-729 methyl Confirmation ion	Q1/Q3 345.0/250.1

For each sample and standard calculate the confirmation ratio:

$$\text{Confirmation Ratio} = \left(\frac{\text{peak area m/z 345.0/250.1}}{\text{peak area m/z 345.0/285.1}} \right)$$

Confirmation of the presence of the analyte is indicated when the retention time of the samples matches that of the standards and the confirmation ratio is in the range of ±20% of the average found for the standards.

A standard curve was prepared using linear regression analysis with 1/x weighting by plotting the equivalent analyte concentration on the abscissa (x-axis) and the respective quantitation ratio (analyte peak area/ internal standard peak area) on the ordinate (y-axis) as shown in figures 1 to 4.

For example, using linear regression with the XDE-729 methyl data from Figure 1:
Equivalent concentration in sample (ng/mL) =

$$\left(\frac{\text{XDE - 729 quantitation ratio} - \text{intercept}}{\text{slope}} \right) \times \text{dilution factor}$$

Convert the concentration from ng/mL of XDE-729 methyl found in the final sample prepared for analysis to µg/mL of methyl accounting for the original sample aliquot as follows:

$$\text{XDE-729 methyl (gross µg/mL)} = \text{ng/mL} \times \frac{\left(\frac{(\text{Ext vol, mL}) \times (\text{Final vol, mL})}{(\text{Samp vol, mL}) \times (\text{Aliq factor, mL})}\right) \times \text{Dil}}{1000}$$

$$\text{XDE-729 methyl (gross µg/mL)} = \text{ng/mL} \times \frac{\left(\frac{(10\,\text{mL}) \times (1\,\text{mL})}{(10\,\text{mL}) \times (10\,\text{mL})}\right) \times}{1000} 1$$

where:

Extraction volume (initial) = 10 mL
Final volume of sample = 1.0 mL
Sample volume (initial) = 10 mL
Aliquot factor = 10 mL
Dilution factor = 1 (unless further dilution is needed)

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

$$\text{Recovery (\%)} = \left(\frac{\text{Concentration found}}{\text{Concentration added}}\right) \times 100$$

An example calculation, using reagent blank, a control aliquot and a LOQ fortified aliquot is demonstrated below.

Example for Calculation

The calculation is exemplified with the ground water specimen 110718-001-0001A10, fortified with 0.50 ug/L (equivalent to 0.0005 µg/mL) of XDE-729 methyl, XDE-729 acid, X11406790 and X11406757. Example is given for XDE-729 methyl calculation:

$$\text{Equivalent concentration in sample (ng/mL)} = \left(\frac{\text{XDE - 729 quantitation ratio} - \text{intercept}}{\text{slope}}\right) \times \text{dilution factor}$$

$$\text{Equivalent concentration in sample (ng/mL)} = \left(\frac{(1540000/76200) - 0.6446}{4.0102}\right) \times 1 = 4.879$$

$$\text{XDE-729 methyl (gross µg/mL)} = \text{ng/mL} \times \frac{\left(\frac{(\text{Ext vol, mL}) \times (\text{Final vol, mL})}{(\text{Samp vol, mL}) \times (\text{Aliq factor, mL})}\right) \times \text{Dil}}{1000}$$

$$\text{XDE-729 methyl (gross µg/mL)} = 4.879\ \frac{\left(\frac{(10\,\text{mL})\times(1\,\text{mL})}{(10\,\text{mL})\times(10\,\text{mL})}\right)\times}{1000}1 = 0.000488$$

where:
Extraction volume (initial) = 10 mL
Final volume of sample = 1.0 mL
Sample volume (initial) = 10 mL
Aliquot factor = 10 mL
Dilution factor = 1 (unless further dilution is needed)

$$\text{Recovery (\%)}=\left(\frac{\text{Concentration found}}{\text{Concentration added}}\right)\times 100\ ;\ \text{Recovery (\%)}=\left(\frac{0.000488\,\text{ug/mL}}{0.00050\,\text{ug/mL}}\right)\times 100 = 98$$

Confirmation of Residue Identity

The method is selective for the determination of XDE-729 methyl, XDE-729 acid, X11406790 and X11406757 by virtue of the chromatographic separation and selective detection system used. Confirmation of the presence of XDE-729 methyl, XDE-729 acid, X11406790 and X11406757 in water specimens are made by comparison of retention times (liquid chromatography) of recovery samples with the retention times of the calibration standards as well as by monitoring two characteristic MS/MS transitions by tandem mass spectrometry, as follows:

Compound	Peak Area, m/z (transitions)
XDE-729 methyl Quant	345.0/285.1
XDE-729 methyl Conf	345.0/250.1
XDE-729 acid Quant	330.8/250.0
XDE-729 acid Conf	330.8/235.0
X'757 Quant	317.0/236
X'757 Conf	317.0/270.9
X'790 Quant	331.0/236.0
X'790 Conf	331.0/271.0

According to published guidelines(5), when detection is performed using tandem mass spectrometry methods, confirmation of the presence of the analyte requires the observation of a precursor ion representing the intact molecule plus two structurally significant product ions observed at the same retention time. The confirmation peak area ratios of the two characteristic MS/MS transitions for each sample should fall within the range of ± 20% of the average

confirmation peak area ratios found for the standards within each sample set.

$$\text{Percent Difference} = [(\text{sample confirmation ratio} \div \text{standards mean confirmation ratio}) - 1] \times 100$$

Examples of confirmation ratio calculation and its percent difference evaluation are showed on tables 13-17.

Stability of Solutions and Extracts

Sample extracts were analyzed as set 110718_S1_rep1 on 02-Dec-2011. Following injection, vials were resealed and placed in a refrigerator for 6 days. On 08-Dec-2011, selected samples were re-injected as set Stability. The recoveries for each analyte and for each matrix were compared for each date of injection. Samples are said to be stable if the mean recoveries are still within the required range (70-120 %).
These samples were found to support stability of each analyte and for each matrix for up to 6 days following preparation for analysis when stored under refrigerated conditions to fully demonstrate stability (as shown in the table 19).

Determination of Matrix Effect

The matrix effects that the sample extracts have upon the analytes during analysis were determined. Matrix effects were evaluated by preparation of matrix-matched standards (for each matrix type) to be used for comparison with standards prepared in a neat solvents. Matrix matched standards are prepared by taking untreated control sample matrix for each matrix type through the cleanup procedure, then fortifying the resulting samples with a known amount of the analyte just prior to injection. Experiments should demonstrate that matrix effects are not significant (i.e. < 20% enhancement or suppression). The matrix effect results for all water specimens are discussed in the results section.

Statistical Treatment of Data

The mean recoveries for the fortified samples were calculated using the “AVERAGE” function of the Microsoft Excel spreadsheet computer program, which divides the sum of the selected cells by the number of determinations. The standard deviation of the recoveries for a fortification level of one matrix type was calculated using the “STDEV” function of the same spreadsheet

program, which sums the squares of the individual deviations from the mean, divides by the number of degrees of freedom, and extracts the square root of the quotient. Percent relative standard deviation, % RSD, was calculated by dividing the standard deviation by the mean, and then multiplying by 100.

Supplemental Notes

1. The instrumental conditions for HPLC and mass spectrometer may be modified to obtain optimal chromatographic separation and sensitivity.
2. Aliquots and final volumes may be adjusted accordingly sample throughput.
3. Due to solubility issues, 20 µg/mL is the maximum concentration suggested for this compound in Methanol.

Assay Time

A typical analytical set would consist of a minimum of six calibration standards encompassing the expected range of sample concentrations, a reagent blank, a control (a non-fortified sample), a minimum of three fortified controls (one of which must be at the LOQ), and 18 samples. This typical analytical run requires approximately 4 hours to prepare a sample set, followed by the chromatographic analysis of 5 instrument-hours. Evaluation of the resulting data requires approx. 3 person-hours.

Thus, the time period from the sample preparation until completion of instrumental analysis, including evaluation, amounts to approx. 8 person-hours or one calendar day.

If analysis is not started immediately as an overnight run, refrigerate sample.

Experiments should demonstrate that matrix effects are not significant (i.e. < 20% enhancement or suppression). Negative values of % matrix effects indicate suppression while positive values indicates enhancement.

Standardization of SPE Elution Profile

There is a possibility that variation in the Phenomenex Strata-X SPE plates may influence the elution profile of XDE-729 methyl. If it is necessary to obtain an elution profile for each lot of SPE columns used to ensure optimum recovery and clean-up efficiency, the following procedure can be used:

1. Place a Strata-X reversed-phase SPE column (3 mL/60 mg) on the vacuum manifold.
2. Condition the SPE column with 3.0 mL of methanol followed by 3.0 mL of water/formic acid (100/0.1, v/v). Dry the SPE column under full vacuum for 5 seconds after each condition step.
3. Transfer the entire sample volume to the SPE column. Draw the sample through the column at a flow rate of approximately 1.0 mL/min. Discard the flow through.
4. Rinse the sample vial with 1.0 mL of water/formic acid (100/0.1, v/v) and load onto the column. Draw the solvent through the column at a flow rate of approximately 1.0 mL/min. Discard the flow through. Dry the SPE column under full vacuum for 5 seconds after the rinse step.
5. Elute the analytes from the SPE column with 3x 500 µL aliquots of methanol. Collect the eluate in clean 15 mL centrifuge tubes.
6. Add 100 µL of 50 ng/mL mixed internal standard to the eluate. Evaporate the sample to near dryness using a TurboVap evaporator with the water bath temperature set at 40 °C and the pressure at approximately 7 psi.
7. Reconstitute sample with 1.0 mL of acetonitrile/water/formic acid (10/90/0.1, v/v/v). Vortex the sample for a few seconds, then sonicate for 2-3 minutes. Vortex a few seconds again to mix well.
8. Transfer approximately 1.0 mL of the sample into the 2.0 mL glass autosampler vial for the LC/MS/MS analysis.
9. Analyze the samples by liquid chromatography with positive-ion electrospray ionization mass spectrometry as described in the instrumental conditions section.

10. Calculate the percent recovery as described in the Calculations section.

11. A typical elution profile for a fortified water sample at 0.50 µg/L is illustrated in table 18. If the elution profile differs from that shown, adjust the volume of methanol to be collected in Step 5.