Method Validation Study for the Determination of Residues of Methoxyfenozide and Its A-ring Phenol Metabolite and B-ring Mono Acid Metabolite in Soil and Sediment Using Liquid Chromatography with Tandem Mass Spectrometry

INTRODUCTION

Scope

This method is applicable for the quantitative determination of residues of methoxyfenozide and its A-ring phenol metabolite and B-ring mono acid metabolite in soil (clay loam and silt loam) and sediment (loamy sand and sand). The method was validated over the concentration range of 0.010-1.0 µg/g with a validated limit of quantitation of 0.010 µg/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, OPPTS 850.7100 (*1*). The validation will also comply 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 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 methoxyfenozide and its A-ring phenol metabolite and B-ring mono acid metabolite are extracted twice from samples by vortex mixing and shaking with a 90% methanol/10% 0.1N hydrochloric acid extraction solution. Both extracts from the centrifuged sample are transferred to the same graduated mixing cylinder (or 50-mL polypropylene graduated centrifuge tube equipped with cap) and the volume is adjusted to 40.0 mL using the 90% methanol/10% 0.1N hydrochloric acid extraction solution. The mixing cylinder (or 50-mL polypropylene graduated centrifuge tube) is capped and the sample is mixed well. An aliquot of the extract is diluted with water. Then the sample is purified following an offline reversed-phase solid-phase extraction (SPE) procedure. Methoxyfenozide and its A-ring phenol and B-ring mono acid metabolites are eluted from the Strata -X polymeric sorbent reversed phase SPE cartridge with two 750-µL aliquots of acetonitrile. The sample eluate is evaporated to dryness and reconstituted with 1.0 mL of a 70% water/30% acetonitrile solution containing 0.1% formic acid. The final sample is analyzed for methoxyfenozide and its A-ring phenol metabolite and B-ring mono acid metabolite by liquid chromatography coupled with positive-ion electrospray ionization (ESI) with tandem mass spectrometry (LC-MS/MS).

Test Substances/Reference Compounds/Analytical Standards

Test Substance	TSN	Percent Purity	Recertification Date	Reference
methoxyfenozide	TSN104129	99.9%	17-Oct-2014	FAPC 10-275236
A-ring phenol	TSN103265	99%	03-Sep-2013	FAPC 09-202030
B-ring mono acid	TSN029592-0001	99%	11-Jul-2012	FAPC 10-274917

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

EXPERIMENTAL

Sample Origin, Numbering, Preparation, Storage, and Characterization

The test system was untreated control soil and sediment samples originating from the Dow AgroSciences Soils Database which were obtained through 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 was included in the study file.

Soils for submission into the Dow AgroSciences Soils Database were prepared for analysis by sieving through a 2mm sieve to remove debris. Sediment samples received no preparation prior to analysis. Soil and sediment samples were stored refrigerated in order to maintain their viability for other studies. A portion of the soils and sediments used for method validation were transferred from the Dow AgroSciences Soils Database to the Dow AgroSciences LLC Sample Management Group where they were weighed into individual tubes for analysis. Soil and sediment samples were stored deep-frozen (at approximately -20 °C) except when removed from the freezer for preparation for the method validation study. Physicochemical characteristics for the soils used are listed in Table 2. Complete documentation for the soil sources may be found in the study file.

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.

methoxyfenozide	*m/z* Q1/Q3 369.1/313.2 (quantitative) *m/z* Q1/Q3 369.1/149.2 (confirmatory)
A-ring phenol	*m/z* Q1/Q3 355.0/299.2 (quantitative) *m/z* Q1/Q3 355.0/135.2 (confirmatory)
B-ring mono acid	*m/z* Q1/Q3 399.1/343.1 (quantitative) *m/z* Q1/Q3 399.1/149.2 (confirmatory)

In order to generate a standard curve, plot the analyte concentration on the abscissa (x-axis) and the respective peak area on the ordinate (y-axis) in Analyst. Using regression analysis, determine the equation for the curve with respect to the abscissa. Refer to Figures 4-9 for example calibration plots and to Figures 10-15 for example calculations. Individual calibration data and set parameters can be found in Tables 3-8.

Determination of Soil Moisture in Field Samples

Residues of methoxyfenozide and its A-ring phenol metabolite and B-ring mono acid metabolite in soil and sediment field samples should be reported on a dry weight basis. Moisture in the samples must be determined prior to analysis. Accurately weigh a 10.0 g portion of soil into a pre-weighed aluminum weighing dish. Place the sample in an oven at 110 °C and allow it to dry for a minimum of 16 hours. Remove the sample from the oven and place in a desiccator containing DRIERITE desiccant. Weigh the sample again when it has cooled to room temperature.

The equation to calculate % moisture follows:

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

where:
w = moisture content, %
W_1 = weight of container and moist soil
W_2 = weight of container and oven dry soil
W_c = weight of container

Determination of Dry Weight Concentrations in Soil

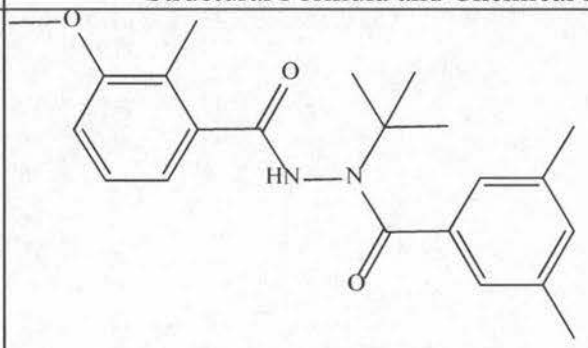
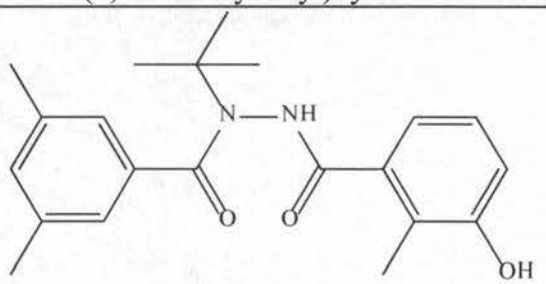
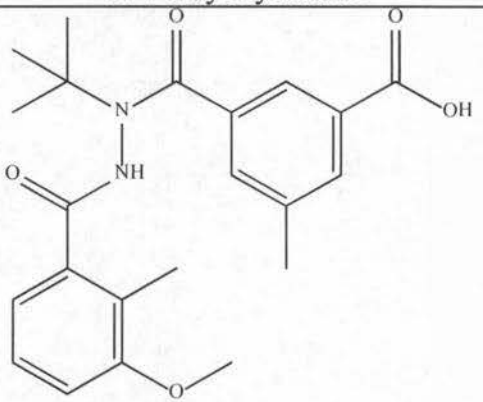
Field sample residues are typically reported on a dry-weight basis. The residue concentrations (ng/g) expressed on a dry-weight basis are calculated as follows.

$$\text{Reported ng/g} = (\text{net ng/g}) * (1 + \frac{\text{percent (\%) moisture}}{100})$$

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 Methoxyfenozide and its A-ring Phenol Metabolite and B-ring Mono Acid Metabolite

Common Name	Structural Formula and Chemical Name
Methoxyfenozide Molecular Formula: $C_{22}H_{28}N_2O_3$ CAS Number: 161050-58-4 Molecular Weight: 368.48	 3-methoxy-2-methylbenzoic acid 2-(3,5-dimethylbenzoyl)-2-(1,1-dimethylethyl)hydrazide
A-ring Phenol Metabolite Molecular Formula: $C_{21}H_{26}N_2O_3$ CAS Number: 252720-16-4 Molecular Weight: 354.45	 *N*'-(3-hydroxy-2-methylbenzoyl)-N-(3,5-dimethylbenzoyl)-*N*-*t*-butyl hydrazine
B-ring Mono Acid Metabolite Molecular Formula: $C_{22}H_{26}N_2O_5$ CAS Number: Unavailable Molecular Weight: 398.46	 (3-({1-*tert*-butyl-2-[(3-methoxy-2-methylphenol)carbonyl]hydrazinyl}-carbonyl)-5-methylbenzoic acid)

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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 and hydrochloric 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 BB2440, Mettler-Toledo, Inc.

Centrifuge, with rotor to accommodate 8 oz wide-mouth bottles, Model Legend XFR, Thermo International Equipment Company

TurboVap LV evaporator, model number ZW-700, Zymark.

Pipet, positive-displacement, 10-100 µL capacity, Model F148504G, Gilson Inc.

Pipet, positive-displacement, 100-1000 µL capacity, Model F148506G, Gilson Inc.

Pipetter, adjustable, Eppendorf, 10-100 µL capacity, catalog number 05-402-48, Brinkmann Instruments.

Pipetter, adjustable, Eppendorf, 50-1000 µL capacity, catalog number 21-378-83, Brinkmann Instruments.

Pipetter, adjustable, Eppendorf, 1000-5000 µL capacity, catalog number 22-46-134-6, Brinkmann Instruments.

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

Vortex mixer, Model G-560, Scientific Industries, Inc.

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

Chromatographic System

Column, analytical, Gemini 5µ C18 110A, 50.0 cm x 2.00 mm, 5-µm particle size, part number 00B-4435-B0, Phenomenex.

Guard column, SecurityGuard cartridge for Gemini C18 HPLC columns with 2.0 to 3.0 mm ID, part number AJ0-7596, Phenomenex.

Guard cartridge holder, part number KJ0-4282, Phenomenex.

Liquid chromatograph, Model 1100, Agilent Technologies.

Mass spectrometer, Model API 4000, AB/Sciex.

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

Glassware and Materials

Bottle, 500 mL, media bottle, catalog number 06-423-3C, Fisher Scientific.

Bottle, 1.0 L, media bottle, catalog number 06-423-3D, Fisher Scientific.

Collection plate, 96-deep well, 2-mL, catalog number 121-5203, International Sorbent Technology Ltd.

Collection plate sealing cap, catalog number 121-5205, Biotage.

Column, SPE, Strata-X 33 um polymeric sorbent, 30 mg sorbent, 1 mL reservoir, catalog number 8B-S100-TAK, Phenomenex.

Cylinder, graduated, 250-mL, catalog number 08-553F, Fisher Scientific.

Cylinder, graduated, 500-mL, catalog number 08-553C, Fisher Scientific.

Cylinder, graduated mixing, 50-mL, catalog number 08-531C, Fisher Scientific.

Cylinder, graduated mixing, 2000-mL, catalog number 08-531H, Fisher Scientific.

Flask,Volumetric, 100 mL, catalog number 10-209D, Fisher Scientific.

Flask,Volumetric, 500 mL, catalog number 10-209G, Fisher Scientific.

Flask, volumetric, 1000-mL, catalog number 10-209H, Fisher Scientific.

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

Pipet tip, positive-displacement, 50 µL capacity, catalog number CP50, Gilson Inc.

Pipet, volumetric, 0.5 mL, catalog number 13-650-2A, Fisher Scientific.

Pipet, volumetric, 1.0 mL, catalog number 13-650-2B, Fisher Scientific.

Pipet, volumetric, 2.0 mL, catalog number 13-650-2C, Fisher Scientific.

Pipet, volumetric, 3.0 mL, catalog number 13-650-3D, Fisher Scientific.

Pipet, volumetric, 5.0 mL, catalog number 13-650-2F, Fisher Scientific.

Pipet, volumetric, 7.0 mL, catalog number 13-650-3H, Fisher Scientific.

Tube, centrifuge tube, polypropylene, 50-mL, number 05-539-9, Fisher Scientific.

Tube, culture, 12 mL, 16 x 100 mm with screw cap, catalog number 99449-16, Corning Products.

Vial, 8-mL, with PTFE-lined screw cap, catalog number 60940A 8, Kimble/Kontes.

Reagents

Acetonitrile, ChromaSolv for HPLC gradient grade, ≥99.9%, catalog number 439134-4L, Sigma-Aldrich.

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

Hydrochloric acid, 1.0 N, certified concentration, catalog number SA48-1, Fisher Scientific.

Methanol, ChromaSolv for HPLC gradient grade, ≥99.9%, catalog number 34885-4L-R, Sigma Aldrich.

Nitrogen, refrigerated liquid, catalog number LQNI, BOC Gases.

Water, ChromaSolv for HPLC gradient grade, ≥99.9%, catalog number 270733-4L, Sigma Aldrich.

Prepared Solutions

acetonitrile + 0.1% formic acid (mobile phase B)

Pipet 2.0 mL of formic acid (96%) into a 2-L graduated mixing cylinder. Dilute to volume with HPLC grade acetonitrile. Cap the cylinder and invert several times to mix the solution prior to use.

50% methanol /50% water (v/v) (port #1 autosampler injection needle wash solution)

Using a 500-mL graduated cylinder, measure 500 mL of HPLC grade water and transfer this to a 1.0 L bottle. Using a 500-mL graduated cylinder, measure 500 mL of methanol and transfer this to the same 1.0 L bottle. Cap the bottle and mix well by shaking, and allow the solution to equilibrate to room temperature before use.

90% methanol /10% 1.0 N hydrochloric acid (v/v) (sample extraction solution)

Using a 2000-mL graduated cylinder, measure 1800 mL of methanol and transfer to a 2.0 L bottle. Using a 250-mL graduated cylinder, measure 200 mL of 1.0 N hydrochloric acid and add to the same 2.0 L bottle containing the methanol. Cap the bottle and mix well by shaking, and allow the solution to equilibrate to room temperature before use.

water + 0.1% formic acid (mobile phase A)

Pipet 2.0 mL of formic acid (96%) into a 2-L graduated mixing cylinder. Dilute to volume with HPLC grade water. Cap the cylinder and invert several times to mix the solution prior to use.

70% water/30% acetonitrile containing 0.1% formic acid (v/v) (calibration standard diluents and final sample diluent)

Using a 500-mL graduated cylinder, measure 500 mL of HPLC grade water and transfer to a 1000-mL volumetric flask. Using a 500-mL graduated cylinder, measure 300 mL of acetonitrile and transfer to the same 1000-mL volumetric flask. Pipet 1.0 mL of formic acid (96%) into the same volumetric flask. Mix well by inverting the flask multiple times, and allow the solution to equilibrate to room temperature. Adjust to volume with HPLC grade water and invert the flask to mix well again before use.

60% water/40% methanol containing 0.1% formic acid (v/v) (SPE wash solution)

Using a 250-mL graduated cylinder, measure 250 mL of HPLC grade water and transfer to a 500-mL volumetric flask. Using a 250-mL graduated cylinder, measure 200 mL of methanol and transfer to the same 1000-mL volumetric flask. Pipet 0.5 mL of formic acid (96%) into the same volumetric flask. Mix well by inverting the flask multiple times, and allow the solution to equilibrate to room temperature. Adjust to volume with HPLC grade water and invert the flask to mix well again before use.

Preparation of Stock and Spiking Solutions

1. Weigh 0.1000 g of methoxyfenozide, RH-2485 (RH-112485) and quantitatively transfer to a 100-mL volumetric flask with acetonitrile. Dilute to volume with acetonitrile to obtain a stock solution containing 1000 µg/mL of methoxyfenozide.

2. Weigh 0.1000 g of the A-ring phenol metabolite, (RH-117236) and quantitatively transfer to a 100-mL volumetric flask with acetonitrile. Dilute to volume with acetonitrile to obtain a stock solution containing 1000 µg/mL of the A-ring phenol metabolite.

3. Weigh 0.1000 g of the B-ring mono acid metabolite, (RH-131154) and quantitatively transfer to a 100-mL volumetric flask with acetonitrile. Add approximately 50 mL of acetonitrile to the flask plus 5 mL of HPLC grade water. Mix well and equilibrate to room temperature. Dilute to volume with acetonitrile to obtain a stock solution containing 1000 µg/mL of the B-ring mono acid metabolite.

4. Pipet 10.0 mL of each of the 1000-µg/mL standard solutions prepared in Step 1 through 3 above and quantitatively transfer each into the same 100-mL volumetric flask. Add approximately 60 mL of HPLC grade water and mix well. Equilibrate to room temperature and dilute to volume with HPLC grade water to obtain a mixed spiking solution containing 100.0 µg/mL of each analyte in a 70% water/30% acetonitrile solution.

5. Pipet 10.0 mL of the 100-µg/mL mixed standard solution prepared in Step 4 above into a 100-mL volumetric flask. Dilute to volume using a 70% water/30% acetonitrile solution containing 0.1% formic acid to obtain a 10.0-µg/mL mixed calibration stock solution.

6. Pipet 10.0 mL of the 10-µg/mL mixed standard solution prepared in Step 5 above into a 100-mL volumetric flask. Dilute to volume using a 70% water/30% acetonitrile solution containing 0.1% formic acid to obtain a 1.0-µg/mL mixed calibration stock solution.

7. Pipet 10.0 mL of the 1.0-µg/mL mixed standard solution prepared in Step 6 above into a 100-mL volumetric flask. Dilute to volume using a 70% water/30% acetonitrile solution containing 0.1% formic acid to obtain a 0.10-µg/mL mixed calibration stock solution.

8. Pipet 10.0 mL of the 0.10-µg/mL mixed standard solution prepared in Step 7 above into a 100-mL volumetric flask. Dilute to volume using a 70% water/30% acetonitrile solution containing 0.1% formic acid to obtain a 0.01-µg/mL mixed calibration stock solution.

Preparation of Calibration Standards

Prepare calibration standards by diluting the appropriate mixed calibration standard stock solutions (Steps 6-8) using a 70% water/30% acetonitrile solution containing 0.1% formic acid according to the following table:

Concentration of Stock Solution	Aliquot of Stock Solution	Final Soln. Volume	Calibration Soln. Final Conc.	Equivalent Sample Conc.[a]
µg/mL	mL	mL	ng/mL	µg/g
1.00	7.5	100	75.0	1.25
1.00	5.0	100	50.0	0.830
1.00	3.5	100	35.0	0.581
1.00	2.0	100	20.0	0.332
1.00	1.0	100	10.0	0.166
0.10	5.0	100	5.00	0.083
0.10	1.0	100	1.00	0.0166
0.01	5.0	100	0.50	0.0083
0.01	1.5	100	0.15	0.0024
0.01	1.0	100	0.10	0.0016

[a]The equivalent sample concentration of methoxyfenozide, the A-ring phenol metabolite, and the B-ring mono acid metabolite is based on taking a 1.0-mL initial aliquot of the 40-mL sample extract, diluting it with 2.0 mL of water, purifying one third of the diluted extract on an SPE cartridge, and reconstituting the eluate to a final volume of 1.0 mL with using a 70% water/30% acetonitrile solution containing 0.1% formic acid (equivalent to 0.04167 g of soil matrix per mL of final sample as prepared for assay).

Sample Preparation

Prepare soil or sediment field samples for analysis by freezing the sample with dry ice or liquid nitrogen and then grinding or chopping with a hammer mill equipped with a 3/16-inch screen size. Soil or sediment samples should be stored deep-frozen prior to analysis.

Analytical Procedure

1. Weigh 5.0 ± 0.05 g of each soil or sediment sample into individual 45-mL vials equipped with PTFE-lined caps (or 50-mL polypropylene graduated centrifuge tubes equipped with caps). (Acceptable stopping point if sample is kept frozen.)

2. For recovery samples, add appropriate aliquots of the mixed spiking solutions to obtain concentrations ranging from 0.01-1.0 µg/g for methoxyfenozide, the A-ring phenol metabolite and the B-ring mono acid metabolite. Refer to table below for example fortification levels to obtain this concentration range.

Sample Description	Spiking Volume µL	Spiking Solution µg/mL	Fortification Level µg/g[a]
CONTROL	---	---	---
LOD	15	1.00	0.003
LOQ	50	1.00	0.010
MID (10 x LOQ)	50	10.0	0.100
HIGH (100 x LOQ)	50	100.0	1.00

[a]Based on a 5.0-g initial soil or sediment sample.

3. Add 20 mL of the 90% methanol/10% 1.0N hydrochloric acid extraction solution to the sample vial.

4. Cap the sample vial with a PTFE-lined cap, pulse vortex mix for about 10 seconds, and shake the sample on a reciprocating flat bed shaker for at approximately 180 excursions/minute for a minimum of 30 minutes.

5. Centrifuge the sample for approximately 5 minutes at approximately 2000 rpm.

6. Transfer the extract to a 50-mL graduated mixing cylinder (or 50-mL polypropylene graduated centrifuge tube equipped with cap may be substituted).

7. Add an additional 15 mL of the 90% methanol/10% 0.1N hydrochloric acid extraction solution to the original sample, pulse vortex mix for about 10 seconds, and shake for an additional 30 minutes (approximate) on a reciprocating flat bed shaker at approximately 180 excursions/minute. (Critical Step: Ensure that the soil or sediment plug is broken up well by vortex mixing or manually shaking before placing the samples on the flat bed shaker.)

8. Centrifuge the sample for 5 minutes at 2000 rpm and combine the second extract with the first extract (Step 6) in the 50-mL graduated mixing cylinder (or 50-mL polypropylene graduated centrifuge tube equipped with cap).

9. Adjust the volume of the combined extract in the centrifuge tube to 40.0 mL using the 90% methanol/10% 0.1N hydrochloric acid extraction solution. Stopper the cylinder (50-mL polypropylene graduated centrifuge tube) and mix thoroughly. (Acceptable stopping point if sample is kept refrigerated.)

10. Using a positive-displacement pipet, transfer 1.0 mL of the sample solution into a 8-mL (2-dram) vial.

11. Dilute the sample from Step 10 with 2.0 mL of HPLC grade water and vortex mix for approximately 5 seconds.

12. Purify samples using the following SPE procedure (each new lot of SPE cartridges should undergo profiling prior to use):

    a. Place a Phenomenex Strata -X polymeric sorbent reversed phase SPE cartridge (30-mg, 1-mL, catalog number 8B-S100-TAK) on a vacuum manifold box.

    b. Condition the SPE cartridge with 1 mL of methanol followed by 1 mL of water, discarding the eluates. Apply full vacuum (approximately -15 to -25 inches of Hg) for about 10 seconds between solvent additions.

    c. Transfer 1.0 mL of the sample solution from Step 11 to the SPE cartridge. Pull the sample through the cartridge at approximately 1 mL/min, discarding the eluate. Dry the plate under full vacuum for 10 seconds after sample has eluted.

    d. Wash the SPE cartridge with 1 mL of a 60% water/40% methanol/0.1% formic acid solution, discarding the eluate. Dry the cartridge under full vacuum for 5 minutes.

    e. Elute the methoxyfenozide and the two metabolites from the SPE cartridge at approximately 1 mL/min with two 750-µL aliquots of acetonitrile, collecting the eluate in a 12-mL (16 x 100 mm) culture tube. Apply full vacuum (approximately -15 to -25 inches of Hg) for about 10 seconds between solvent additions.

13. Evaporate the acetonitrile to dryness at approximately 40 °C using nitrogen (approximately 10 psi) on a TurboVap evaporator.

14. Reconstitute the samples in 1.0 mL of 70% water/30% acetonitrile solution containing 0.1% formic acid. Pulse vortex for about 10 seconds to mix well.

15. Transfer a portion of each sample that is ready for analysis to a 96-deep well plate.

16. Add approximately 1 mL of each of the calibration standards to the same plate and seal the plate. (Acceptable stopping point if sample is kept refrigerated.)

17. Analyze the calibration standards and samples by positive-ion ESI LC/MS/MS, injecting the calibration standards interspersed with the samples throughout the run.

18. Calculate the percent recovery found for each analyte.

14. Analyze the samples along with the calibration standards using the LC/MS/MS conditions

listed. Determine the suitability of the chromatographic system using the following criteria:

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

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

c. Appearance of chromatograms: Visually determine that the chromatograms resemble those shown in Figures 19-29 with respect to peak response, baseline noise, and background interference. Visually determine that a minimum signal-to-noise ratio of at least 10:1 has been attained for the 0.5-ng/mL calibration standard (equivalent to 0.008 µg/g of methoxyfenozide, the A-ring phenol metabolite and the B-ring mono acid metabolite in the soil or sediment sample).

15. If any sample concentrations exceed the range of the standard calibration curve, re-analyze the sample after diluting using the 70% water/30% acetonitrile solution containing 0.1% formic acid. The analyte concentration should be 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. Electronic pipets are used only for pipetting aqueous solutions. If they are used for pipetting non-aqueous solutions, the pipets should be calibrated following the manufacturer's instruction manual and Standard Operating Procedures (1).

3. Different volumes of solutions may be prepared when carrying out the analytical method as long as the same ratios are maintained.

4. Based on availability of material, weighing of the analytical standard can be modified and the subsequent solution preparation scheme adjusted.

5. The instrumental conditions may be modified to obtain optimal chromatographic separation and sensitivity.

6. Other types of regression models (either linear or non-linear) may be applied to give the best fit, correlation coefficient (r), for the data where this can be fully justified based on the detection system used.

Instrumental Conditions

Typical HPLC Operating Conditions

Instrumentation:	Agilent Model 1100 autosampler Agilent Model 1100 binary pump Agilent Model 1100 degasser AB/Sciex API 4000 LC/MS/MS System AB/Sciex Analyst 1.5.1 data System
Column:	Gemini C18 110A, 2.00 x 50 mm, 5.0-µm (SecurityGuard cartridge for Gemini C18 HPLC column with 2.0 to 3.0 mm ID)
Column Temperature:	Ambient (approximately 22 °C)
Injection Volume:	30 µL
Injection Wash Program:	(Autosampler loop and needle washed with) 1) 700 µL of a 50% MeOH/50% water (no valve wash) 2) 2 x 700 µL of MeOH (no valve wash) 3) 2 x 700 µL of water (no valve wash)
Mobile Phase:	A –water containing 0.1% formic acid B –acetonitrile containing 0.1% formic acid
Flow Rate:	0.40 mL/min, flow diverted to source after 1.0 min. (approx 250 µL split to source)

Gradient:

Time, min	Solvent A, %	Solvent B, %
0.0	70	30
1.00	70	30
8.00	10	90
9.00	70	30
12.00	70	30

Flow Diverter

Flow to Waste	0.0 min → 1.0 min
Flow to Source	1.0 min → 8.0 min
Flow to Waste	8.0 min → end of run

Equilibration Time: 3.0 minutes

Typical Mass Spectrometry Operating Conditions

Interface:	Turbo Spray
Polarity:	Positive
Scan Type:	MRM
Resolution:	Q1 – unit, Q3 – unit
Curtain Gas (CUR):	30
Collision Gas (CAD):	6
Temperature (TEM):	350°C
Ion Source Gas 1 (GS1):	50
Ion Source Gas 2 (GS2):	50

Period 1

Pre-acquisition Delay:	0.0 min
Acquisition Time:	8.0 min
IonSpray Voltage (IS):	5000 volts
Entrance Potential (EP):	10 volts

Analytes:	Precursor Ion Q1 *m/z*	Product Ion Q3 *m/z*	Dwell Time (ms)	Collision Energy (CE)	Declustering Potential (DP)	Cell Exit Potential (CXP)
Methoxyfenozide (quantification)	369.1	313.2	150 ms	11 V	46 V	12 V
Methoxyfenozide (confirmation)	369.1	149.2	150 ms	23 V	46 V	18 V
A-ring phenol (quantification)	355.0	299.2	150 ms	11 V	66 V	10 V
A-ring phenol (confirmation)	355.0	135.2	150 ms	23 V	66 V	12 V
B-ring mono acid (quantification)	399.1	343.1	150 ms	11 V	76 V	12 V
B-ring mono acid (confirmation)	399.1	149.2	150 ms	21 V	76 V	12 V