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Determination of Residues of Aminopyralid in Soil by Liquid Chromatography with Tandem Mass Spectrometric Detection

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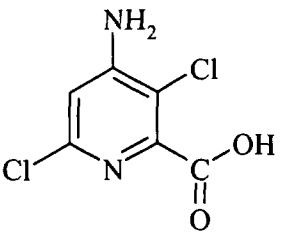
1. SCOPE

This method is applicable for the quantitative determination of residues of aminopyralid (4-amino-3,6-dichloro-2-pyridinecarboxylic acid) in soil. The method was validated over the concentration range of 0.001-0.20 µg/g with a validated limit of quantitation of 0.001 µg/g.



Aminopyralid
CAS No. 150114-71-9

Common and chemical names, molecular formulas, and the nominal masses for the above structure and related compounds are given in Table 1.

2. PRINCIPLE

Residues of aminopyralid are extracted from soil by shaking with a methanol/2 N sodium hydroxide (90:10) solution. After centrifugation, the solution is poured off and the extraction is performed again. The second extraction solution is then combined with the first and brought to a fixed volume. During the extraction, bound residues and base-labile conjugates are hydrolyzed to yield free aminopyralid.

An aliquot of the solutions are then evaporated to dryness and reconstituted in water. The sample is then purified using a mixed-mode polymeric anion-exchange solid-phase extraction (SPE) plate. After elution from the SPE plate with an ethyl acetate/trifluoroacetic acid (99:1) solution, a stable-isotope labeled internal standard ($^{13}C_2{}^2H^{15}N$-aminopyralid) is added, and the eluate is then evaporated to dryness. The residue is reconstituted in an acetonitrile/pyridine/1-butanol (22:2:1) solution, and derivatized with butyl chloroformate to form the 1-butyl esters (1-BE) of the analyte and internal standard. After derivatization, the mixture is diluted with a methanol/water (40:60) with 0.05% formic acid and 5 mM ammonium formate solution and then

analyzed by liquid chromatography with positive-ion electrospray 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, 1-butanol, butyl chloroformate, ethyl acetate, methanol, and pyridine are flammable and should be used in well-ventilated areas away from ignition sources.

3.3. Acetic acid, butyl chloroformate, formic acid, sodium hydroxide, and trifluoroacetic acid are corrosive and toxic and can cause severe burns. It is imperative that proper eye and personal protection equipment be worn when handling these reagents.

3.4. Pyridine is a possible carcinogen. It is imperative that proper eye and personal protection equipment be worn when handling this reagent.

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 PG2002, Mettler-Toledo, Inc.

4.1.3. Centrifuge, with rotor to accommodate 40-mL vials, Model Centra-GP8, Thermo International Equipment Company, Needham Heights, MA 02494.

4.1.4. Desiccator, 250-mm i.d., catalog number 08-595E, Fisher Scientific, Pittsburgh, PA 15219.

4.1.5. Dispenser, adjustable bottle-top, 10-50-mL volume, catalog number 13-688-144, Fisher Scientific.

4.1.6. Evaporator, N-Evap, Model 111, Organomation Associates, Inc., South Berlin, MA 01503.

4.1.7. Evaporator, SPE Dry Dual 96-well, Argonaut Technologies, Redwood City, CA 94063.

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

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

4.1.10. Pipet, electronic, Eppendorf, 100-5000 µL, catalog number 022461346, Brinkmann Instruments, Inc., Westbury, NY 11590. (Note 12.2.)

4.1.11. Pipet, positive displacement, Pos-D, 200-1000 µL, catalog number MR-1000, Gilson, Inc., Middleton, WI 53562.

4.1.12. Pipet, positive displacement, Pos-D, 10-100 µL, catalog number MR-100, Gilson, Inc.

4.1.13. Pipet, positive displacement, Pos-D, 0.5-10 µL, catalog number MR-10, Gilson, Inc.

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

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

4.1.16. Vacuum manifold, 96-well, catalog number 121-9601, International Sorbent Technology Ltd, Hengoed, Mid Glamorgan UK and distributed by Argonaut Technologies, 1101 Chess Drive, Foster City, CA 94404.

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

4.2. Chromatographic System

4.2.1. Column, analytical, Zorbax SB-C8, 4.6 x 75 mm, 3.5-µm particle size, catalog number 7995208-344, Agilent Technologies, Wilmington, DE 19808.

4.2.2. Liquid chromatograph autosampler, Model 1100, Agilent Technologies.

4.2.3. Liquid chromatograph binary pump, Model 1100, Agilent Technologies.

4.2.4. Liquid chromatograph column oven, Model 1100, Agilent Technologies.

4.2.5. Liquid chromatograph vacuum degasser, Model 1100, Agilent Technologies.

4.2.6. Mass spectrometer, Model API 3000, Applied Biosystems, Foster City, CA 94404.

4.2.7. Mass spectrometer data system, Model Analyst 1.4.2, Applied Biosystems.

5. GLASSWARE AND MATERIALS (Note 12.1.)

5.1. Cap, 96-well piercable sealing, catalog number 121-5204, Argonaut Technologies.

5.2. Collection plate, polypropylene 96-well, 2 mL/well, catalog number AH0-7194, Phenomenex, Inc., Torrance, CA 90501.

5.3. Cylinder, graduated mixing, 50-mL, catalog number 08-564-5C, Fisher Scientific.

5.4. Cylinder, graduated mixing, 2000-mL, catalog number 08-564-5E, Fisher Scientific.

5.5. Dish, aluminum weighing, catalog number 08-732, Fisher Scientific.

5.6. Flask, volumetric, 10-mL, catalog number 10-209A, Fisher Scientific.

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

5.8. Flask, volumetric, 200-mL, catalog number 10-209E, Fisher Scientific.

5.9. Flask, volumetric, 250-mL, catalog number 10-209F, Fisher Scientific.

5.10. Pipet tips, Eppendorf, 100-5000 µL, catalog number 022491989, Brinkmann Instruments, Inc.

5.11. Pipet tips, Pos-D, 200-1000 µL, catalog number CP-10, Gilson, Inc.

5.12. Pipet tips, Pos-D, 10-100 µL, catalog number CP-100, Gilson, Inc.

5.13. Pipet tips, Pos-D, 0.5-10 µL, catalog number CP-1000, Gilson, Inc.

5.14. Pipet, transfer, 5.75-in, catalog number 13-678-20B, Fisher Scientific.

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

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

5.17. Pipet, volumetric, 10-mL, catalog number 13-650-2L, Fisher Scientific.

5.18. Pipet, volumetric, 15-mL, catalog number 13-650-2M, Fisher Scientific.

5.19. Pipet, volumetric, 20-mL, catalog number 13-650-2N, Fisher Scientific.

5.20. Pipet, volumetric, 25-mL, catalog number 13-650-2P, Fisher Scientific.

5.21. Pipet, volumetric, 30-mL, catalog number 13-650-2Q, Fisher Scientific.

5.22. Pipet, volumetric, 80-mL, catalog number 13-650-2S, Fisher Scientific.

5.23. Pipet, volumetric, 100-mL, catalog number 13-650-2U, Fisher Scientific.

5.24. Plate, 96-well SPE, Oasis MAX, 30-mg, catalog number 186000373, Waters, Milford, MA 01757.

5.25. Vial, 8-mL, with PTFE-lined screw cap, catalog number 03-340-60B, Fisher Scientific.

5.26. Vial, 12-mL, with PTFE-lined screw cap, catalog number 03-340-60C, Fisher Scientific

5.27. Vial, 40-mL, with PTFE-lined screw cap, catalog number 03-971-7G, Fisher Scientific.

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

6.1. Reagents

6.1.1. Acetic acid, glacial, ACS plus grade, catalog number A38S-500, Fisher Scientific.

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

6.1.3. Ammonium formate, HPLC grade, catalog number A666-500, Fisher Scientific.

6.1.4. 1-Butanol, 99.8% purity, catalog number 28,154-9, Sigma-Aldrich, St. Louis, MO 63178.

6.1.5. Butyl chloroformate, 98% purity, catalog number 18,446-2, Sigma-Aldrich.

6.1.6. Dessicant, Drierite adsorbent, catalog number 24001, W. A. Hammond Drierite Company, Xenia, OH 45385.

6.1.7. Ethyl acetate, OmniSolv grade, catalog number EX-0241-1, EMD Chemicals.

6.1.8. Formic acid, 95%, ACS reagent grade, catalog number F0507-100ML, Sigma-Aldrich.

6.1.9. Methanol, ChromAR HPLC grade, catalog number 3041, Mallinckrodt Baker Inc.

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

6.1.11. Pyridine, 99.9+% purity, catalog number 27,040-7, Sigma-Aldrich.

6.1.12. Sodium hydroxide, 2.0 N, ACS reagent grade, certified concentration, catalog number SS264-1, Fisher Scientific.

6.1.13. Trifluoroacetic acid, 99+% purity, catalog number 30,203-1, Sigma-Aldrich.

6.1.14. Water, HPLC grade, catalog number WX0004-1, EMD Chemicals.

6.2. Standards

6.2.1. aminopyralid (4-amino-3,6-dichloro-2-pyridinecarboxylic acid)

Obtain from Test Substance Coordinator, Dow AgroSciences LLC, 9330 Zionsville Road, Building 304, Indianapolis, IN 46268-1054.

6.2.2. $^{13}C_2{}^{2}H^{15}N$-aminopyralid (4-amino-3,6-dichloropicolinic acid-1-^{15}N-2,6-^{13}C-5-*d*)

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

6.3. Prepared Solutions

6.3.1. derivatization coupling reagent — acetonitrile/pyridine/1-butanol (22:2:1) (v/v/v)

Pipet 10.0 mL of 1-butanol and 20.0 mL of pyridine into a 250-mL volumetric flask containing approximately 150 mL of acetonitrile. Swirl the flask, and allow to equilibrate to room temperature. Dilute to volume with acetonitrile.

6.3.2. ethyl acetate/trifluoroacetic acid (99:1) (v/v)

Pipet 2.0 mL of trifluoroacetic acid into a 200-mL volumetric flask containing approximately 100 mL of ethyl acetate. Swirl the flask, and dilute to volume with ethyl acetate.

6.3.3. methanol/2 N sodium hydroxide (90:10) (v/v)

Add 1800 mL methanol to a 2-L graduated mixing cylinder. Add approximately 150 mL of 2 N sodium hydroxide, swirl, and allow to equilibrate to room temperature. Dilute to volume with 2 N sodium hydroxide, swirl, and mix well.

6.3.4. methanol/water/acetic acid (50:49:1) (v/v/v)

Pipet 100 mL of methanol and 2.0 mL of acetic acid into a 200-mL volumetric flask containing approximately 50 mL of water. Swirl the flask, and allow to equilibrate to room temperature. Dilute to volume with water.

6.3.5. methanol with 0.05% formic acid and 5 mM ammonium formate

Weigh 0.63 g of ammonium formate into a 40-mL vial and quantitatively transfer with 100 mL methanol to a 2-L graduated mixing cylinder containing approximately 1800 mL of methanol. Add 1.0 mL of formic acid, dilute to volume with methanol, and mix well.

6.3.6. water with 0.05% formic acid and 5 mM ammonium formate

Weigh 0.63 g of ammonium formate into a 40-mL vial and quantitatively transfer with 100 mL HPLC water to a 2-L graduated mixing cylinder containing approximately 1800 mL of water. Add 1.0 mL of formic acid, dilute to volume with water, and mix well.

6.3.7. water/methanol (60:40) with 0.05% formic acid and 5 mM ammonium formate

Pipet 80 mL of methanol with 0.05% formic acid and 5 mM ammonium formate (Section 6.3.5.) into a 200-mL volumetric flask containing approximately 80 mL of water with 0.05% formic acid and 5 mM ammonium formate (Section 6.3.6.). Swirl the flask, and allow to equilibrate to room temperature. Dilute to volume with water with 0.05% formic acid and 5 mM ammonium formate (Section 6.3.6.).

7. PREPARATION OF STANDARDS

7.1. Preparation of Aminopyralid Spiking Solutions for Samples

7.1.1. Weigh 0.1000 g of aminopyralid analytical standard and quantitatively transfer to a 100-mL volumetric flask with acetonitrile. Dilute to volume with acetonitrile to obtain a 1000-µg/mL stock solution.

7.1.2. Prepare solutions for spiking samples by diluting the solution from Section 7.1.1 with acetonitrile as follows:

Concentration of Stock Solution	Aliquot Of Stock Solution	Final Solution Volume	Concentration of Spiking Solution	Equivalent Sample Concentration[a]
µg/mL	mL	mL	µg/mL	µg/g
1000	10.0	100	100.0	10.0
100	10.0	100	10.0	1.00
10.0	20.0	100	2.00	0.20
10.0	10.0	100	1.00	0.10
1.00	10.0	100	0.10	0.01
0.10	10.0	100	0.01	0.001
0.01	30.0	100	0.003	0.0003

[a] The equivalent sample concentration is based on fortifying a 5.0-g sample with 500 µL of spiking solution.

7.2. Preparation of the $^{13}C_2{}^2H^{15}N$-Aminopyralid Internal Standard Solution

7.2.1. Accurately weigh 0.0025 g of $^{13}C_2{}^2H^{15}N$-aminopyralid standard and quantitatively transfer to a 100-mL volumetric flask with acetonitrile. Dilute to volume with acetonitrile to obtain a 25.0-µg/mL stock solution.

7.2.2. Using a volumetric pipet, dispense 1.0 mL of the 25.0-µg/mL solution in Section 7.2.1 into a 200-mL volumetric flask and dilute to volume with acetonitrile to obtain a 0.125-µg/mL (125-ng/mL) stock solution.

7.3. Preparation of Aminopyralid 1-Butyl Ester Calibration Standards for Quantitation

7.3.1. Using a positive displacement pipet, dispense aliquots of the 0.01-10.0-µg/mL spiking solutions from Section 7.1.2 into a series of 10-mL volumetric flasks and dilute to volume with derivatization coupling reagent solution (Section 6.3.1.). The concentration of the calibration solutions are as shown in the fourth column in the following table:

Concentration of Spiking Solution	Aliquot Of Spiking Solution	Final Solution Volume	Concentration of Calibration Solution	Concentration of Derivatized Calib. Standard	Equivalent Sample Concentration
µg/mL	mL	mL	ng/mL	a.e. ng/mL	µg/g
0.01	0.150	10.0	0.150	0.030	0.0003
0.01	0.500	10.0	0.500	0.100	0.0010
0.10	0.125	10.0	1.25	0.250	0.0025
0.10	0.250	10.0	2.50	0.500	0.0050
0.10	0.500	10.0	5.00	1.00	0.010
1.00	0.125	10.0	12.5	2.50	0.025
1.00	0.250	10.0	25.0	5.00	0.050
1.00	0.500	10.0	50.0	10.0	0.100
1.00	1.000	10.0	100.0	20.0	0.200
10.0	0.125	10.0	125.0	25.0	0.250

7.3.2. Prepare calibration standards with each sample set by dispensing 80 µL of the internal standard solution containing 125 ng/mL of $^{13}C_2{}^2H^{15}N$-aminopyralid (Section 7.2.2.) into a 96-well plate.

7.3.3. Evaporate the internal standard solution to dryness using an SPE Dual Dry evaporator set at 40 °C and a nitrogen flow rate of approximately 50 L/min.

7.3.4. Using a positive displacement pipet, dispense 200 µL of the 0.15-125.0-ng/mL calibration solutions from Section 7.3.1 into the series of wells used in Step 7.3.2, and derivatize according to the procedure described in Section 9.3.24-9.3.26. The final concentration range of these calibration standards is from 0.030-25.0 ng/mL, as shown in the fifth column above.

7.4. Preparation of the Aminopyralid 1-Butyl Ester Standard to Determine Isotopic Crossover

7.4.1. Using a positive displacement pipet, dispense 100 µL of the 0.10-µg/mL aminopyralid solution in Section 7.1.2 into an 8-mL vial and derivatize according to the procedure described in Section 9.3.22-9.3.27. The resulting solution contains aminopyralid 1-butyl ester equivalent to 10.0 ng/mL of aminopyralid.

7.5. Preparation of the $^{13}C_2{}^2H^{15}N$-Aminopyralid 1-Butyl Ester Standard to Determine Isotopic Crossover

7.5.1. Using a positive displacement pipet, dispense 80 µL of the 125-ng/mL $^{13}C_2{}^2H^{15}N$-aminopyralid solution in Section 7.2.2 into an 8-mL vial and derivatize according to the procedure described in Section 9.3.22-9.3.27. The resulting solution contains $^{13}C_2{}^2H^{15}N$-aminopyralid 1-butyl ester equivalent to 10.0 ng/mL of $^{13}C_2{}^2H^{15}N$-aminopyralid.

8. INSTRUMENTAL CONDITIONS

8.1. Typical HPLC Operating Conditions (Note 12.3.)

Instrumentation:	Agilent Model 1100 autosampler Agilent Model 1100 binary pump Agilent Model 1100 degasser MDS/Sciex API 3000 LC/MS/MS System MDS/Sciex Analyst 1.4.2 data system
Column:	Zorbax SB-C8 4.6 x 75 mm, 3.5-µm
Column Temperature:	35 °C
Injection Volume:	35 µL
Run Time:	8.0 minutes
Mobile Phase:	A – methanol with 0.05% formic acid and 5 mM ammonium formate B – water with 0.05% formic acid and 5 mM ammonium formate
Flow Rate:	900 µL/min (approx 200 µL/min split to source)

Gradient:

Time, min	Solvent A, %	Solvent B, %
0.0	40	60
1.0	40	60
6.0	100	0
8.0	100	0

Flow Diverter Program:	1) 0.0 to 4.5 min: flow to waste 2) 4.5 to 6.5 min: flow to source 3) 6.5 to 7.0 min: flow to waste
Equilibration Time:	3.0 minutes

8.2. Typical Mass Spectrometry Operating Conditions (Note 12.3.)

Interface:	Electrospray
Scan Type:	MRM
Resolution:	Q1 – unit, Q3 – unit
Nebulizer Gas (NEB):	13
Curtain Gas (CUR):	9
Collision Gas (CAD):	7
Temperature (TEM):	425 °C
Ion Source Gas 1 (GS1):	8 psi
Ion Source Gas 2 (GS2):	7000 mL/min
Declustering Potential:	34 volts
Focusing Potential:	170 volts
Entrance Potential:	9 volts
IonSpray Voltage (IS):	1300 volts
Pre-acquisition Delay:	0.0 minutes
Acquisition Time:	7.0 minutes
Polarity:	Positive

Analytes:	Precursor Ion Q1	Product Ion Q3	Collision Time, ms	Cell Exit Potential	Collision Energy
aminopyralid BE (quantitation)	263.1	134.1	150	10	57
aminopyralid BE (confirmation 1)	263.1	161.1	150	12	39
aminopyralid BE (confirmation 2)	263.1	189.0	150	14	25
$^{13}C_2{}^2H^{15}N$-aminopyralid BE	269.1	194.9	150	14	27

8.3. Mass Spectra

Full-scan and product-ion mass spectra of aminopyralid 1-butyl ester showing the $(M+H)^+$ at *m/z* 263 and the product ions at *m/z* 134, *m/z* 161, and *m/z* 189 are illustrated in Figure 1. Full-scan and product-ion mass spectra of $^{13}C_2{}^2H^{15}N$-aminopyralid 1-butyl ester showing the $(M+H)^+$+2 at *m/z* 269 and the product ion at *m/z* 195 are illustrated in Figure 2.

8.4. Typical Calibration Curve

A typical calibration curve for the determination of aminopyralid in soil is shown in Figure 3.

8.5. Typical Chromatograms

Typical chromatograms of a standard, a control sample, a 0.001-µg/g (LOQ) recovery sample, and a 0.20-µg/g recovery sample for the determination of aminopyralid in soil are illustrated in Figures 4-13.

9. DETERMINATION OF RECOVERY OF AMINOPYRALID FROM SOIL

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 limit of detection.
At least two controls fortified at the limit of quantitation.
At least two controls fortified at the expected residue concentration in the samples.

9.2. Sample Preparation

Prepare soil samples for analysis by freezing with dry ice or liquid nitrogen and then grinding or chopping using a hammer mill with a 1/8 -inch screen size. Prepared soil samples should be stored frozen at approximately -10 to -20 °C until analysis.

9.3. Sample Analysis

9.3.1. Weigh 5.0 ± 0.05 g of the soil sample into a 40-mL vial with PTFE-lined cap.

9.3.2. For preparing fortified samples, add 500-µL aliquots of the appropriate spiking solutions (Section 7.1.2.) to encompass the necessary concentration range.

9.3.3. Add 25 mL of a methanol/2 N sodium hydroxide extraction solution (90:10) to the sample vial and cap with a PTFE-lined screw cap.

9.3.4. Vortex mix the sample for approximately 30 seconds.

9.3.5. Place samples on a reciprocating shaker at approximately 180 excursions/minute for 1 hour.

9.3.6. Remove the sample from the shaker and centrifuge the sample at approximately 2000 rpm for 5 minutes.

9.3.7. Decant the extract into a 50-mL graduated mixing cylinder with a glass stopper.

9.3.8. Add an additional 15 mL of the methanol/2 N sodium hydroxide extraction solution (90:10) to the vial containing the soil sample.

9.3.9. Cap the vial and vortex mix the sample for approximately 30 seconds to ensure that the soil plug has been re-suspended in the extraction solvent.

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

9.3.11. Remove the sample from the shaker and centrifuge the sample at approximately 2000 rpm for 5 minutes.

9.3.12. Combine the extract from Step 9.3.11 with the original extract from Step 9.3.7, by decanting the extract into the graduated mixing cylinder containing the original extract.

9.3.13. Bring the soil extract to a 40-mL final volume with the addition of the methanol/2 N sodium hydroxide extraction solution (90:10).

9.3.14. Cap the mixing cylinder and invert several times to ensure homogeneity of the sample extract.

9.3.15. Pipet a 1.0-mL aliquot of the soil extract into a new 12-mL glass vial.

9.3.16. Evaporate the extract to dryness using an N-Evap evaporator set at approximately 40 °C with a nitrogen gas flow rate of approximately 500 mL/min.

9.3.17. Reconstitute the residue in 1.25 mL of HPLC water and cap the vial with a PTFE-lined closure.

9.3.18. Sonicate the sample for approximately 1 minute and vortex mix the sample for approximately 10 seconds.

9.3.19. Depending on the expected aminopyralid concentration in the samples, add an additional amount of HPLC water to the 12-mL vial. The following table can be used as a guide for sample size selection as well as for the amount of water to be added. Cap the sample vial and vortex mix for 1-2 seconds. If no dilution is required, this step may be skipped. If the concentration of the field samples is unknown, prepare samples according to the 0.001-0.20 µg/g range (no dilution).

Concentration Range	Additional Water Added	Final Volume	Dilution Factor
µg/g	µL	µL	
0.008-1.60	8750	10000	8
0.004-0.80	3750	5000	4
0.002-0.40	1250	2500	2
0.001-0.20	0	1250	1

9.3.20. Purify the sample using the following SPE procedure (Section 11.4.):

a. Place a Waters Oasis MAX SPE plate on the vacuum manifold.

b. Condition the SPE well with 1 mL of methanol followed by 1 mL of HPLC water. (Dry the SPE plate under full vacuum (approximately -10 inches Hg) for 5 seconds between solvents.)

c. Transfer 1.0 mL of the sample solution from Step 9.3.19 to the SPE column. Draw the sample through the well at a flow rate of approximately 1 mL/min, discarding the eluate.

d. Wash the SPE well with two 1-mL aliquots of HPLC water. Draw the solvent through the well at a flow rate of approximately 1 mL/min, discarding the eluate.

e. Rinse the SPE well with a 1.0-mL aliquot of a methanol/water/acetic acid solution (50:49:1). Draw the solvent through the well at a flow rate of approximately 1 mL/min, discarding the eluate. Dry the SPE plate for 15 minutes under full vacuum (approximately -10 inches Hg).

f. Elute the aminopyralid from the SPE well with three 500-µL aliquots of an ethyl acetate/trifluoroacetic acid solution (99:1), collecting the eluate in a 2-mL deep 96-well collection plate.

9.3.21. Add 80 µL of the internal standard solution containing 125 ng/mL of $^{13}C_2{}^{2}H^{15}N$-aminopyralid (Section 7.2.2.) to the wells containing the SPE eluate. (See Section 7.3.2 for concurrent calibration standard preparation)

9.3.22. Evaporate the sample eluate to dryness using a SPE Dual Dry evaporator set at 40 °C and a nitrogen flow rate of approximately 50 L/min.

9.3.23. Add 200 µL of the derivatization coupling reagent (Section 6.3.1.) to the sample wells.

9.3.24. Derivatize the sample (Section 9.3.23.), the calibration standards (Section 7.3.4.), and the crossover standards (Sections 7.4.1 and 7.5.1, if needed) by pipetting 10 µL of butyl chloroformate derivatizing reagent into the wells.

9.3.25. Gently vortex the plate for 5 minutes to mix and allow the mixture to react.

9.3.26. Add 790 µL of the water/methanol (60:40) solution containing 0.05% formic acid and 5 mM ammonium formate to the sample wells. Gently vortex mix for approximately one minute, and firmly seal the plate with a cap.

9.3.27. Analyze the crossover standards (Sections 7.4.1 and 7.5.1, if needed), calibration standards (Section 7.3.4.), and samples by HPLC with positive-ion electrospray tandem mass spectrometry as described in Section 8. Determine the suitability of the chromatographic system using the following performance criteria:

a. Standard curve linearity: Determine that the correlation coefficient equals or exceeds 0.995 for the least squares equation which describes the detector response as a function of standard curve concentration. If power regression is used, the power exponent should be between 0.90-1.10.

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

c. Appearance of chromatograms: Visually determine that the chromatograms resemble those shown in Figures 4-13 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 analyte in the 0.10-ng/mL calibration standard.

9.3.28. If any sample concentrations exceed the range of the standard calibration curve, reanalyze the sample using the appropriate dilution factor. The table in Step 9.3.19 may be used for guidance.

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 aminopyralid and $^{13}C_2{}^{2}H^{15}N$-aminopyralid, inject the derivatized crossover standards described in Sections 7.4.1 and 7.5.1, and determine the peak areas for the analyte and internal standard as indicated below.

aminopyralid 1-BE	Q1/Q3 *m/z* 263/134
$^{13}C_2{}^{2}H^{15}N$-aminopyralid 1-BE	Q1/Q3 *m/z* 269/195

For example, to determine the contribution of the unlabeled aminopyralid to the labeled $^{13}C_2{}^{2}H^{15}N$-aminopyralid internal standard:

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

$$\text{Crossover Factor (analyte}\rightarrow\text{ISTD)} = \frac{\text{peak area at } m/z \text{ 269/195}}{\text{peak area at } m/z \text{ 263/134}}$$

In a similar manner, to determine the contribution of the labeled $^{13}C_2{}^{2}H^{15}N$-aminopyralid internal standard to the unlabeled aminopyralid:

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

$$\text{Crossover Factor (ISTD}\rightarrow\text{ analyte)} = \frac{\text{peak area at } m/z \text{ 263/134}}{\text{peak area at } m/z \text{ 269/195}}$$

During method development, no 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 (Note 12.4.)

10.2.1. Inject the series of calibration standards as described in Sections 7.3.2-7.3.4 and determine the peak areas for the analyte as indicated below.

Aminopyralid	Q1/Q3 *m/z* 263/134 (quantitation) Q1/Q3 *m/z* 263/161 (confirmation 1) Q1/Q3 *m/z* 263/189 (confirmation 2)
$^{13}C_2{}^{2}H^{15}N$-aminopyralid	Q1/Q3 *m/z* 269/195

10.2.2. For each standard, calculate the aminopyralid quantitation ratio.

For example, using the data for the aminopyralid 0.1-ng/mL standard from Figures 3 and 4:

$$\text{Quantitation Ratio} = \frac{\text{peak area of quantitation ion}}{\text{peak area of internal standard ion}}$$

$$\text{Quantitation Ratio} = \frac{\text{aminopyralid peak area at Q1/Q3 } m/z \text{ 263/134}}{\text{ISTD peak area at Q1/Q3 } m/z \text{ 269/195}}$$

$$\text{Quantitation Ratio} = \frac{8587}{1218207}$$

$$\text{Quantitation Ratio} = 0.0070$$

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 Figure 3. Using linear regression analysis (13.3.) with a 1/x weighting (13.4.), determine the equation for the curve with respect to the abscissa.

For example, using the aminopyralid data from Figure 3:

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

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

$$\text{aminopyralid conc. (ng/mL)} = \left(\frac{\text{aminopyralid quantitation ratio} - (0.0000)}{0.0610}\right)$$

10.3. Calculation of Percent Recovery

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

For example, using the data for aminopyralid recovery in the sandy loam soil sample 020-0001 + 0.001 µg/g A3 from Figure 6:

$$\text{aminopyralid gross (ng/mL)} = \left(\frac{\text{aminopyralid quantitation ratio} - (0.0000)}{0.0610}\right)$$

$$\text{aminopyralid gross (ng/mL)} = \left(\frac{0.0046 - 0.0000}{0.0610}\right)$$

$$\text{aminopyralid (gross)} = 0.0754 \text{ ng/mL}$$

10.3.2. Convert the concentration (ng/mL) of aminopyralid found in the final sample prepared for analysis to µg/g of aminopyralid in the original sample as follows:

$$\begin{aligned}
\text{aminopyralid (gross µg/g)} &= 0.0754\ \text{ng/mL} \times \left(\frac{(\text{Extraction Vol} \times \text{Final Vol})}{(\text{Aliquot Vol} \times \text{Nominal Weight})}\right) \times \text{dilution factor} \\
\text{aminopyralid (gross µg/g)} &= 0.0754\ \text{ng/mL} \times \left(\frac{(40.0\ \text{mL} \times 1.0\ \text{mL})}{(0.8\ \text{mL} \times 5\ \text{g})}\right) \times \frac{1.00\ \mu\text{g}}{1000\ \text{ng}} \times 1 \\
\text{aminopyralid (gross)} &= 0.00075\ \mu\text{g/g}
\end{aligned}$$

10.3.3. Determine the net concentration in each recovery sample by subtracting any signal found in the control sample from that of the gross analyte concentration.

For example, using the data for control sandy loam soil 020-0001 A1 in Figure 5, and continuing with data for the sandy loam soil recovery sample 020-0001 + 0.001 µg/g A3 in Figure 6:

$$\begin{aligned}
\text{aminopyralid net (µg/g)} &= \text{aminopyralid (gross µg/g)} - \text{aminopyralid (control µg/g)} \\
\text{aminopyralid net (µg/g)} &= 0.00075 - 0.0000 \\
\text{aminopyralid (net)} &= 0.00075\ \mu\text{g/g}
\end{aligned}$$

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

$$\begin{aligned}
\text{Recovery} &= \frac{\text{Concentration Found}}{\text{Concentration Added}} \times 100\% \\
\text{Recovery} &= \frac{0.00075\ \mu\text{g/g}}{0.0010\ \mu\text{g/g}} \times 100\% \\
\text{Recovery} &= 75\%
\end{aligned}$$

Note that the precision in these calculations is reduced by rounding at each step as was shown above for demonstration purposes.

10.4. Determination of Aminopyralid in Soil Samples

10.4.1. Determine the gross concentration of the analyte in each treated sample by substituting the respective quantitation ratio into the equation for the standard calibration curve and calculating the uncorrected residue result as described in Sections 10.3.1-10.3.2.

10.4.2. For those analyses that require correction for method recovery, use the average recovery of all the recovery samples for that matrix fortified at or above the limit of quantitation from a given sample set to correct for method efficiency.

For example, using the data for aminopyralid from Figure 6 and Table 2 for the soil recovery samples analyzed with set 071121 S07 on 26-Oct-2007:

$$\begin{pmatrix}\text{aminopyralid}\\(\text{corrected}\,\mu g/g)\end{pmatrix} = \begin{pmatrix}\text{aminopyralid}\\(\text{gross}\,\mu g/g)\end{pmatrix} \times \left(\frac{100}{\text{Avg \% Recovery}}\right)$$

$$\begin{pmatrix}\text{aminopyralid}\\(\text{corrected}\,\mu g/g)\end{pmatrix} = 0.00075\ \mu g/g \times \frac{100}{77}$$

$$\begin{pmatrix}\text{aminopyralid}\\(\text{corrected})\end{pmatrix} = 0.00097\ \mu g/g$$

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 dessicator 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:

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

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

10.6. Determination of Dry Weight Concentrations of Aminopyralid in Soil

10.6.1. Determine the analyte concentrations in the sample as described in Sections 10.4.1-10.4.2.

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:

$$\begin{array}{c}\text{aminopyralid conc.} \\ \text{(dry weight µg/g)}\end{array} = \begin{array}{c}\text{aminopyralid conc.} \\ \text{(µg/g)}\end{array} \times \left(1 + \frac{\%\,\text{Moisture}}{100}\right)$$

11.4. Standardization of Waters Oasis MAX Solid-Phase Plate Elution Profile

There is a possibility that variation in the Waters Oasis MAX SPE plates may influence the elution profile of aminopyralid. If it is necessary to obtain an elution profile for the SPE plates used to optimize recovery and clean-up efficiency, the following procedure can be used:

11.4.1. Add 50.0 µL of the 1.00-µg/mL spiking solution from Section 7.1.2 to an 8-mL vial containing 1.0 mL of a methanol/2 N sodium hydroxide (90:10) solution. Evaporate to dryness with an N-Evap evaporator set at 40 °C and a nitrogen flow rate of approximately 500 mL/min.

11.4.2. Reconstitute in 1.25 mL of water. Vortex mix and sonicate for approximately 1 minute.

11.4.3. Place a Waters Oasis MAX SPE plate on the vacuum manifold.

11.4.4. Condition the SPE plate with 1 mL of methanol followed by 1 mL of HPLC water. (Dry the SPE plate under full vacuum, approximately -10 inches Hg, for approximately 5 seconds between solvents.)

11.4.5. Transfer 1.0 mL of the sample solution from Step 11.4.2 to the SPE plate. Draw the sample through the plate at a flow rate of approximately 1 mL/min, discarding the eluate.

11.4.6. Rinse the SPE plate with two 1 mL aliquots of HPLC water. Draw the solvent through the plate at a flow rate of approximately 1 mL/min, discarding the eluate.

11.4.7. Rinse the SPE plate with 1-mL of a methanol/water/acetic acid solution (50:49:1). Draw the solvent through the plate at a flow rate of approximately 1 mL/min, discarding the eluate. Dry the SPE plate for approximately 15 minutes under full vacuum (approximately -10 inches Hg) after the third solvent rinse.

11.4.8. Elute the aminopyralid from the SPE plate with 2.5 mL of an ethyl acetate/trifluoroacetic acid solution (99:1), collecting 0.5-mL aliquots of the eluate in separate 8-mL vials.

11.4.9. For each fraction collected, proceed as described in Sections 9.3.21 through 9.3.27.

11.4.10. Calculate the percent recovery as described in Section 10.3.

A typical elution profile is illustrated in Figure 14. If the elution profile differs from that shown, adjust the volume of the ethyl acetate/trifluoroacetic acid solution (99:1) solution to be collected in Step 9.3.20.f.

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 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. (13.6)

12.3. The LC/MS/MS operating conditions may be modified to obtain optimal chromatographic separation and mass spectrometric performance. Additionally, the assignment of quantitation and confirmation transitions may be exchanged if needed.

12.4. The type of regression model can be chosen to give the best fit for the data.

13. REFERENCES

13.1. Jenden, D. J.; Roch, M.; Booth, R. A. *Anal. Biochem.* **1973**, 55, 438-448.

13.2. Barbalas, M. P.; Garland, W. A., *J. Pharm. Sci.* **1991**, *80*(10), 922-927.

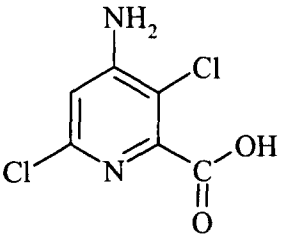
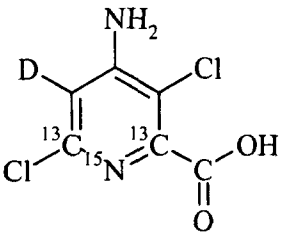
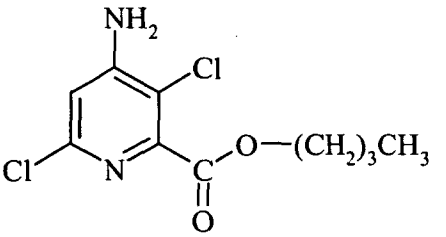
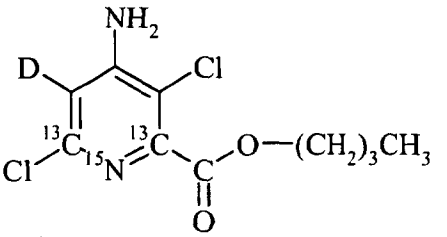
13.3. Freund, J. E.; Williams, F. J. *Dictionary/Outline of Basic Statistics*; Dover: New York, 1991; p 170.

13.4. Neter, J.; Kutner, M. H.; Nachtssheim, C. J. ; Wasserman, W. *Applied Linear Regression Models*; The McGraw-Hill Company: New York, 1996; p 409.

13.5. Keith, L. H.; Crummett, W.; Deegan, J., Jr.; Libby, R. A.; Taylor, J. K.; Wentler, G. *Anal. Chem.* **1983**, *55*, 2210-2218.

13.6. *Standard Operating Procedure for Pipettes*; Brinkmann/Eppendorf SOP 5101-C20, Brinkmann Instruments, Inc., Westbury, NY, 2001.

Table 1. Identity and Structures of Aminopyralid and Related Compounds

Common Name of Compound	Structure and CAS Name
Aminopyralid Molecular Formula: $C_6H_4Cl_2N_2O_2$ Formula Weight 207.02 Nominal Mass: 206 CAS Number: 150114-71-9	 4-amino-3,6-dichloro-2-pyridinecarboxylic acid
$^{13}C_2{}^{2}H^{15}N$-Aminopyralid Molecular Formula: $^{13}C_2C_4{}^{2}HH_3Cl_2{}^{15}NNO_2$ Formula Weight 211.00 Nominal Mass: 210 CAS Number: not available	 4-amino-3,6-dichloro-2-pyridinecarboxylic acid-1-^{15}N-2,6-^{13}C-5-*d*
Aminopyralid 1-Butyl Ester Molecular Formula: $C_{10}H_{12}Cl_2N_2O_2$ Formula Weight 263.12 Nominal Mass: 262 CAS Number: not available	 4-amino-3,6-dichloro-2-pyridinecarboxylic acid, 1-butyl ester
$^{13}C_2{}^{2}H^{15}N$-Aminopyralid 1-Butyl Ester Molecular Formula: $^{13}C_2C_8{}^{2}HH_{11}Cl_2{}^{15}NNO_2$ Formula Weight 267.11 Nominal Mass: 266 CAS Number: not available	 4-amino-3,6-dichloro-2-pyridinecarboxylic acid-1-^{15}N-2,6-^{13}C-5-*d*, 1-butyl ester