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

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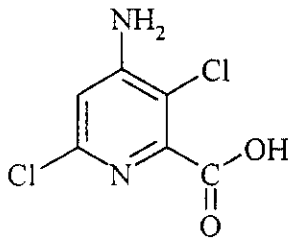
1. SCOPE

This method is applicable for the quantitative determination of residues of aminopyralid (4-amino-3,6-dichloro-2-pyridine carboxylic acid) in soil. The method was validated using four soil types representative of soils found in range and pasture land over the concentration range of 0.0015-0.10 µg/g with a validated limit of quantitation of 0.0015 µg/g.



Aminopyralid
CAS Number 150114-71-9

Common and chemical names, and the corresponding nominal masses for aminopyralid and related compounds are given in Table 1.

2. PRINCIPLE

Residues of aminopyralid are extracted from the soil by shaking with an acetonitrile/1N hydrochloric acid solution (90:10). The sample is centrifuged, and the extract is decanted into a graduated mixing cylinder. A second extraction is performed by adding extraction solution to the soil and shaking the sample on a reciprocating shaker for 30 minutes. The sample is centrifuged and the second extract is combined with the original extract.

An aliquot of the extract is evaporated to dryness and reconstituted in 1N hydrochloric acid. An aliquot of the concentrated extract is purified using a polymeric 96-well solid phase extraction (SPE) plate. The SPE plate is washed with a water/methanol solution (95:5) and eluted with acetonitrile. The eluate is evaporated to dryness, and the residues are reconstituted in an acetonitrile/pyridine/butanol solution (22:2:1). The residue is derivatized with butyl chloroformate and diluted with a methanol/water/acetic acid mobile phase (50:50:0.1). The purified extract is then analyzed by high performance liquid chromatography with positive-ion electrospray (ESI) tandem mass spectrometry (LC/MS/MS).

A calibration curve resulting from the injection of eight standard concentrations demonstrates linearity with a correlation coefficient of at least 0.9996. LC/MS/MS affords a highly specific method for both quantitation and confirmation of residue identity by retention time matching in conjunction with monitoring the MS/MS ion transitions of aminopyralid butyl ester at *m/z* 263.0/134.0 and aminopyralid stable isotope butyl ester at *m/z* 268.0/139.0.

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

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

3.4. Butyl chloroformate and pyridine are toxic. It is imperative that proper eye and personal protection equipment be used when handling all chemicals.

4. EQUIPMENT (Note 12.1.)

4.1. Laboratory Equipment

4.1.1. Balance, analytical, Model AE200, Mettler-Toledo, Inc., Columbus, OH 43240.

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

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

4.1.4. Evaporator, Turbo Vap LV, Zymark Corporation, Hopkinton, MA 01748.

4.1.5. Evaporator, SPE-Dry-96, Argonaut Technologies Inc., Foster City, CA 94404.

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

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

4.1.8. Pipetter, adjustable, Eppendorf, 5-100 µL, catalog number 21-378-74, Fisher Scientific, Pittsburgh, PA 15275.

4.1.9. Pipetter, adjustable, Eppendorf, 20-300 µL, catalog number 21-378-77, Fisher Scientific.

4.1.10. Pipetter, adjustable, Eppendorf, 50-1000 µL, catalog number 21-378-83, Fisher Scientific.

4.1.11. Pipetter, adjustable, Eppendorf, 1000 - 5000 µL, catalog number 21-378-79, Fisher Scientific.

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

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

4.1.14. Vacuum manifold, 96-well, catalog number 121-9601, International Sorbent Technology Ltd., Hengoed, Mid Glamorgan, UK and distributed by Argonaut Technologies, Inc.

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

4.2. Chromatographic System (Note 12.1.)

4.2.1. Column, analytical, Diazem 3000, C18, 4.6 x 100 mm, 3.0-µm, catalog number 030C18Q-10046, Diazem Corporation, Midland, MI 48640.

4.2.2. Liquid chromatograph autosampler, Model 1100, Agilent Technologies, Wilmington, Delaware 19808.

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

4.2.4. Liquid chromatograph degasser, Model 1100, Agilent Technologies.

4.2.5. Mass spectrometer, Model API 3000, MDS/Sciex, Foster City, CA 94404.

4.2.6. Mass spectrometer data system, Analyst 1.1, MDS/Sciex.

5. GLASSWARE AND MATERIALS (Note 12.1.)

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

5.2. Collection plate, 2-mL, catalog number 121-5203, International Sorbent Technology Ltd.

5.3. Collection plate sealing cap, catalog number 121-5205, International Sorbent Technology Ltd.

5.4. Cylinder, graduated mixing, 50-mL, catalog number 08-565C, Fisher Scientific.

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

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

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

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

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

5.10. Flask, volumetric, 50-mL, catalog number 10-206C, Fisher Scientific.

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

5.12. Flask, volumetric, 200-mL, catalog number 10-206E, Fisher Scientific.

5.13. Flask, volumetric, 500-mL, catalog number 10-206G, Fisher Scientific.

5.14. Pipet, disposable polyethylene, catalog number 13-711-7, Fisher Scientific.

5.15. Pipet, 10-mL disposable sterile plugged borosilicate glass, catalog number 13-678-25E, Fisher Scientific.

5.16. Pipet, volumetric, 0.5-mL, catalog number 261-6010, National Scientific Company.

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

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

5.19. Pipet, volumetric, 2.5-mL, catalog number 13-650-2Y, Fisher Scientific.

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

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

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

5.23. Pipet, volumetric, 25.0-mL, catalog number 261-6025, National Scientific Company.

5.24. Pipetter tips, Brinkmann Eppendorf, 1-200-µL tip, catalog number 21-371-3, Fisher Scientific.

5.25. Pipetter tips, Brinkmann Eppendorf, 1000-µL tip, catalog number 22350901, Brinkmann Instruments, Inc., Westbury, NY 11590.

5.26. Pipetter tips, Brinkmann Eppendorf, 5-mL tip, catalog number 22350811, Brinkmann Instruments, Inc.

5.27. SPE 96-well plate, Phenomenex Strata X 33 µm, 30-mg packing, catalog number 8E-S100-TGB, Phenomenex, Torrance, CA 90501.

5.28. Tube, 12-mL culture, 16x100 mm, catalog number 14-961-29, Fisher Scientific.

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

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

6.1. Reagents

6.1.1. Acetic acid, certified ACS Plus grade, catalog number A38S-500, Fisher Scientific.

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

6.1.3. 1-Butanol, certified ACS, grade, catalog number A399-20, Fisher Scientific.

6.1.4. Butyl chloroformate, catalog number 18, 446-2, Sigma-Aldrich, Milwaukee, WI 53201.

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

6.1.6. Methanol, HPLC grade, catalog number 3041, Mallinckrodt Baker, Inc.

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

6.1.8. Pyridine, ACS reagent grade, catalog number 18, 452-7, Sigma-Aldrich.

6.1.9. Water, HPLC grade, catalog number WX0004-1, EM Science, Gibbstown, NJ 08027.

6.2. Standards

6.2.1. aminopyralid: 4-amino-3,6-dichloro-2-pyridine carboxylic acid

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

6.2.2. $^{13}C_2{}^{15}N$-aminopyralid: 4-amino-3,6-dichloro-2-[$^{13}C_2{}^{15}N$] pyridine carboxylic acid

Obtain from Specialty Synthesis Center Of Expertise, Dow AgroSciences LLC, 9330 Zionsville Road, Building 306, Indianapolis, IN 46268. Dow AgroSciences will provide the stable isotope internal standard free of charge.

6.3. Prepared Solutions

6.3.1. acetonitrile/1N hydrochloric acid (90:10)

Pour 1800 mL of acetonitrile into a 2-L graduated mixing cylinder. Measure and add 200 mL of 1N hydrochloric acid to the cylinder. Place a glass stopper on the cylinder and invert several times to mix. Allow the solution to equilibrate to room temperature before use.

6.3.2. acetonitrile/pyridine/butanol (22:2:1)

Add 880 mL of acetonitrile to a 1-L graduated mixing cylinder. Measure 80 mL of pyridine with a graduated cylinder and combine with the acetonitrile. Add 40 mL of 1-butanol to the acetonitrile:pyridine solution using a 50-mL graduated cylinder. Place a glass stopper on the cylinder and invert several times to mix. Allow the solution to equilibrate to room temperature before use.

6.3.3. methanol/acetic acid (100:0.1)

Pipet 1.0 mL of acetic acid into a 1-L graduated mixing cylinder containing 1.0 L of methanol. Place a glass stopper on the cylinder and invert several times to mix. Transfer the solution to a 1-L HPLC solvent bottle.

6.3.4. methanol/water/acetic acid (50:50:0.1)

Combine 125 mL of methanol and 125 mL of water in a 250-mL graduated mixing cylinder. Add 250 µL of acetic acid to the solution. Place a glass stopper on the cylinder and invert several times to mix. Allow the solution to equilibrate to room temperature before use.

6.3.5. water/acetic acid (100:0.1)

Pipette 1.0 mL of acetic acid into a 1-L graduated mixing cylinder containing 1.0 L of water. Place a glass stopper on the cylinder and invert several times to mix. Transfer the solution to a 1-L HPLC solvent bottle.

6.3.6. Water/methanol (95:5 v/v)

Add approximately 80 mL of water to a 100-mL volumetric flask. Pipet 5.0 mL of methanol into the flask. Place a glass stopper on the flask and invert several times to mix. Allow the mixture to equilibrate to room temperature and then bring to volume with water. Place a stopper on the flask and invert several times to ensure homogeneity.

7. PREPARATION OF STANDARDS (Note 12.2.)

7.1. Preparation of Aminopyralid Fortification Solutions

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

7.1.2. Pipet 10.0 mL of the 1000-µg/mL solutions in Section 7.1.1 into a 100-mL volumetric flask and adjust to volume with acetonitrile to obtain a 100.0-µg/mL fortification solution.

7.1.3. Pipet 10.0 mL of the 100.0-µg/mL standard solution in Section 7.1.2 into a 100-mL volumetric flask and adjust to volume with acetonitrile to obtain a 10.0-µg/mL fortification solution.

7.1.4. Pipet 10.0 mL of the 10.0-µg/mL fortification solution in Section 7.1.3 into a 100-mL volumetric flask and adjust to volume with acetonitrile to obtain a 1.0-µg/mL fortification solution.

7.1.5. Pipet 10.0 mL of the 1.0-µg/mL fortification solution in Section 7.1.4 into a 100-mL volumetric flask and adjust to volume with acetonitrile to obtain a 0.10-µg/mL fortification solution.

7.1.6. Pipet 10.0 mL of the 0.10-µg/mL fortification solution in Section 7.1.5 into a 100-mL volumetric flask and adjust to volume with acetonitrile to obtain a 0.01-µg/mL fortification solution.

7.2. Preparation of Aminopyralid Stable Isotope Internal Standard Solution

7.2.1. Weigh 0.005 g of the aminopyralid stable isotope analytical standard and quantitatively transfer to a 50-mL volumetric flask with an acetonitrile/pyridine/butanol (22:2:1) solution. Dilute to volume with the acetonitrile/pyridine/butanol (22:2:1) solution to obtain a nominal 100-µg/mL stock solution.

7.2.2. Pipet 5.0 mL of the nominal 100-µg/mL stable isotope stock solution in Section 7.2.1. into a 500-mL volumetric flask and dilute to volume with an acetonitrile/pyridine/butanol (22:2:1) solution to obtain a nominal 1.0-µg/mL internal standard solution.

7.2.3. Pipet 1.0 mL of the nominal 100-µg/mL stable isotope stock solution in Section 7.2.1. into a 100-mL volumetric flask and adjust to volume with acetonitrile to obtain a nominal 1.0-µg/mL internal standard solution.

7.2.4. Pipet 25.0 mL of the nominal 1.0-µg/mL stable isotope internal standard solution in Section 7.2.3. into a 200-mL volumetric flask and adjust to volume with acetonitrile to obtain a nominal 0.125-µg/mL internal standard solution.

7.3. Preparation of Aminopyralid Calibration Solutions

7.3.1. Pipet 1.0 mL of the 100.0-µg/mL aminopyralid spiking solution from Section 7.1.2 into a 100-mL volumetric flask and adjust to volume with an acetonitrile/pyridine/butanol (22:2:1) solution to obtain a 1.0-µg/mL calibration standard stock solution.

7.3.2. Pipet 10.0 mL of the 1.0-µg/mL calibration solution from Section 7.3.1 into a 100-mL volumetric flask and adjust to volume with an acetonitrile/pyridine/butanol (22:2:1) solution to obtain a 0.1-µg/mL calibration solution.

7.3.3. Pipet 10.0 mL of the 0.1-µg/mL calibration solution from Section 7.3.2 into a 100-mL volumetric flask and adjust to volume with an acetonitrile/pyridine/butanol (22:2:1) solution to obtain a 0.01-µg/mL calibration solution.

7.3.4. Prepare calibration solutions by pipetting 12.5 mL of the nominal 1.0-µg/mL stable isotope internal standard solution (Section 7.2.2) into each 100-mL volumetric flask and then adding the appropriate aliquot of the calibration standard stock solutions from Section 7.3.1-7.3.3 and diluting these with an acetonitrile/pyridine/butanol (22:2:1) solution as described in the table below:

Original Standard Conc.	Aliquot of Original Standard	Final Solution Volume	Calib.Soln. Final Conc.	Calib.Soln. Conc. After Derivatization [a]	Equivalent Sample Conc.[b]
µg/mL	mL	mL	µg/mL		µg/g
1.0	25.0	100.0	0.25	0.05	0.133
1.0	17.5	100.0	0.175	0.035	0.0933
1.0	10.0	100.0	0.10	0.02	0.0533
1.0	5.0	100.0	0.05	0.01	0.0267
1.0	2.5	100.0	0.025	0.005	0.0133
0.10	5.0	100.0	0.005	0.001	0.00267
0.10	2.5	100.0	0.0025	0.0005	0.00133
0.01	5.0	100.0	0.0005	0.0001	0.000267

[a] Aminopyralid Acid Equivalent.
[b] Equivalent sample concentration resulting from taking a 200-µL aliquot of each calibration standard, derivatizing, and diluting to a final volume of 1.0 mL.

7.4. Preparation of Aminopyralid and Aminopyralid Stable Isotope Crossover Standard Solutions

7.4.1. Prepare a 0.125-µg/mL aminopyralid crossover standard solution by pipetting 12.5 mL of the 1.0-µg/mL aminopyralid calibration standard solution (Section 7.3.1.) into a 100-mL volumetric flask and diluting to volume with an acetonitrile/pyridine/butanol (22:2:1) solution.

7.4.2. Prepare a 0.125-µg/mL aminopyralid stable isotope crossover standard solution by pipetting 12.5 mL of the 1.0-µg/mL aminopyralid stable isotope standard solution (Section 7.2.2.) into a 100-mL volumetric flask and diluting to volume with an acetonitrile/pyridine/butanol (22:2:1) solution.

8. LIQUID CHROMATOGRAPHY/TANDEM MASS SPECTROMETRY (LC/MS/MS)

8.1. Typical Liquid Chromatography 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 1.1 data system
Column:	Diazem 3000, C18 4.6 x 100 mm, 3.0-µm
Column Temperature:	35 °C
Injection Volume:	50 µL
Run Time:	6.0 minutes

Mobile Phase:	A –Methanol with 0.1% acetic acid B –Water with 0.1% acetic acid
Flow Rate:	900 µL/min; Flow diverted to source after 3 minutes

Gradient:

Time, min	A, %	B, %
0.0	50	50
5.0	100	0
6.0	100	0

Equilibration Time: 3 minutes

8.2. Typical Mass Spectrometry Operating Conditions

Interface:	TurboIonSpray
Polarity:	Positive
Scan Type:	MRM
Resolution:	Q1 – unit, Q3 – unit
Curtain Gas (CUR):	13 psi
Collision Gas (CAD):	12 psi
Temperature (TEM):	425 °C
Ion Source Gas 1 (GS1):	8 psi
Ion Source Gas 2 (GS2):	7000 cc/min.

Acquisition Time Delay:	3 minutes
Period Duration:	3 minutes
Polarity:	Positive
IonSpray Voltage (IS):	5000

Compound:

	Ion, *m/z*			Collision
	Q1	Q3	Time, ms	Energy
aminopyralid butyl ester	263.0	134.0	150	49
aminopyralid stable isotope butyl ester	268.0	139.0	150	49

8.3. Mass Spectra

Typical mass spectra and product-ion spectra of aminopyralid butyl ester and aminopyralid stable isotope butyl ester are given in Figures 1 and 2, respectively.

8.4. Typical Calibration Curve

A typical calibration curve for the determination of aminopyralid butyl ester in soil is presented in Figure 3.

8.5. Typical Chromatograms

Typical chromatograms of a 0.025-µg/mL aminopyralid stable isotope butyl ester crossover standard and a 0.025-µg/mL aminopyralid butyl ester cross over standard are presented in Figure 4.

Typical chromatograms of a 0.0005-µg/mL calibration standard, a control silt loam sample, a control silt loam sample fortified at 0.0015 µg/g (LOQ), and a control silt loam sample fortified at 0.015 µg/g (10 times the LOQ) are presented in Figure 5.

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

9.2. Sample Preparation

Prepare the samples for analysis by freezing with liquid nitrogen or dry ice and then grinding or chopping using a hammer mill with a 1/8-inch or 3/16-inch screen size. Prepared samples should be stored frozen at approximately –10 to –20 °C prior to 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 recovery samples, add appropriate aliquots of the fortification solution to obtain concentrations ranging from 0.0003 µg/g to 0.10 µg/g. Refer to Table 2 for a suggested fortification scheme.

9.3.3. Add 25 mL of an acetonitrile/1N hydrochloric acid 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 acetonitrile/1N hydrochloric acid 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 acetonitrile/1N hydrochloric acid 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. Using a disposable graduated glass pipet, remove a 6.0-mL aliquot of the soil extract and dispense into a new 40-mL vial.

9.3.16. Evaporate the extract to dryness using a TurboVap®LV evaporator set at approximately 45 °C with a nitrogen gas flow rate of approximately 10 psi.

9.3.17. Reconstitute the residue in 3.0 mL of 1N hydrochloric acid.

9.3.18. Sonicate the sample for approximately 5 minutes and vortex mix the sample for approximately 10 seconds.

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

a. Place a Phenomenex Strata-X 96-well SPE plate (30 mg) on a 96-well vacuum manifold.

b. Condition the 96-well plate with 1.0 mL of methanol followed by 1.0 mL of 1N hydrochloric acid (dry the plate under full vacuum for 5 seconds between solvents).

c. Transfer 1.5 mL of the sample solution from Step 9.3.18 to the 96-well plate. Pull the sample through the plate at approximately 1 mL/min, discarding the eluate. Dry the plate under full vacuum for 5 seconds after the sample has been eluted.

d. Wash the plate with 750 µL of a water/methanol solution (95:5), discarding the eluate. Dry the cartridge under full vacuum for 15 minutes.

e. Elute the aminopyralid from the plate at approximately 1 mL/min with two 500-µL aliquots of acetonitrile, collecting the eluate in a 2-mL deep-well collection plate containing 200 µL of the 0.125 µg/mL aminopyralid stable isotope internal standard solution (Section 7.2.4.).

9.3.20. Evaporate the sample eluate from Section 9.3.19.e, containing internal standard, to dryness with nitrogen using a 96-well evaporator set at approximately 50 °C with a nitrogen flow rate of approximately 500 mL/minute.

9.3.21. Pipet 200 µL of the acetonitrile/pyridine/butanol (22:2:1) solution into each sample well and vortex mix the samples gently for approximately 1 minute.

9.3.22. Pipet 200 µL of each of the calibration standards (Section 7.3.4.) into individual empty wells in the plate containing the samples.

9.3.23. Pipet 200 µL of the 0.125-µg/mL aminopyralid crossover standard (Section 7.4.1.) into an empty well in the collection plate to produce the analyte to ISTD crossover standard.

9.3.24. Pipet 200 µL of the 0.125-µg/mL aminopyralid stable isotope standard solution (Section 7.4.2.) into an empty well to produce the ISTD to analyte crossover standard.

9.3.25. Derivatize the samples (Section 9.3.21.), the calibration standards (Section 9.3.22.), and the crossover standards (Sections 9.3.23. and 9.3.24.) by pipetting 10 µL of butyl chloroformate into each well.

9.3.26. Allow the samples and standards to stand at room temperature for approximately 5 minutes to ensure complete derivatization.

9.3.27. Pipet 790 µL of the methanol/water/acetic acid (50:50:0.1) mobile phase into each well containing the samples and standards.

9.3.28. Cap the 96-well collection plate and gently vortex mix the samples and standards for approximately 30 seconds.

9.3.29. Analyze the butyl derivatives of the crossover standards, the samples, and the calibration standards using the LC/MS/MS conditions listed in Section 8. 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. Appearance of chromatograms: Visually determine that the chromatograms resemble those shown in Figures 5a-5d with respect to peak response, baseline noise, and background interference. Visually determine that a minimum signal-to-noise ratio of 10:1 has been attained for the 0.0025-µg/mL calibration standard (equivalent to 0.0013 µg/g of aminopyralid in the soil sample).

9.3.30. If the sample concentrations exceed the range of the standard calibration curve, dilute the sample with derivatized $^{13}C_2{}^{15}N$-aminopyralid internal standard solution (Section 7.4.2.) to obtain responses within the range of the calibration curve.

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 (13.1, 13.2).

10.1.1. To determine the isotopic crossover for aminopyralid and $^{13}C_2{}^{15}N$-aminopyralid, inject the derivatized crossover standards described in Sections 9.3.23 and 9.3.24 and determine the peak areas for the analyte and internal standards as indicated below.

aminopyralid	*m/z* Q1/Q3 263/134
$^{13}C_2{}^{15}N$- aminopyralid	*m/z* Q1/Q3 268/139

For example, to determine the contribution of the labeled $^{13}C_2{}^{15}N$-aminopyralid internal standard to the unlabeled aminopyralid using the $^{13}C_2{}^{15}N$-aminopyralid data from Figure 4a:

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

$$\text{Crossover Factor (ISTD}\rightarrow\text{analyte)} = \frac{\text{peak area at } m/z \text{ 263/134}}{\text{peak area at } m/z \text{ 268/139}}$$

$$\text{Crossover Factor (ISTD}\rightarrow\text{analyte)} = \frac{142}{77131} = 0.00184$$

In a similar manner, to determine the contribution of the unlabeled aminopyralid to the labeled $^{13}C_2{}^{15}N$-aminopyralid internal standard using the aminopyralid data from Figure 4b:

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

$$\text{Crossover Factor (analyte}\rightarrow\text{ISTD)} = \frac{\text{peak area at } m/z \text{ 268/139}}{\text{peak area at } m/z \text{ 263/134}}$$

$$\text{Crossover Factor (analyte}\rightarrow\text{ISTD)} = \frac{1085}{199962} = 0.00543$$

The crossover factors illustrated above indicate that the (analyte to ISTD) crossover is greater than the (ISTD to analyte) crossover. However, the amount of the $^{13}C_2{}^{15}N$-aminopyralid internal standard used in the method was chosen to minimize the effect of the (analyte to ISTD) crossover contribution over the concentration range of the calibration curve. As a result, the measured quantitation ratio only needs to be corrected for the (ISTD to analyte) crossover contribution.

10.2. Calculation of Standard Calibration Curve

10.2.1. Inject the series of calibration standards described in Section 7.3.4, using the conditions listed in Section 8, and determine the peak areas for the aminopyralid analyte and internal standard as indicated below.

aminopyralid butyl ester	Q1/Q3 *m/z* 263/134
$^{13}C_2{}^{15}N$ aminopyralid stable isotope butyl ester	Q1/Q3 *m/z* 268/139

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

For example, using the data for aminopyralid from Figure 5a:

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

$$\text{Quantitation Ratio (uncorrected)} = \frac{\text{analyte peak area at } m/z \text{ 263/134}}{\text{ISTD peak area at } m/z \text{ 268/139}}$$

$$\text{Quantitation Ratio (uncorrected)} = \frac{4207}{77412}$$

$$\text{Quantitation Ratio (uncorrected)} = 0.05435$$

10.2.3. For each standard, calculate the corrected aminopyralid quantitation ratio, which accounts for the isotopic contribution of the $^{13}C_2{}^{15}N$- aminopyralid internal standard to the aminopyralid.

For example, using the data for aminopyralid from Figure 4a and 5a:

$$\text{Quantitation Ratio (uncorrected)} = \text{Quantitation Ratio (uncorrected)} - \text{Quantitation Ratio (ISTD)}$$

$$\text{Quantitation Ratio (uncorrected)} = 0.05435 - 0.00184$$

$$\text{Quantitation Ratio (corrected)} = 0.05251$$

10.2.4. Prepare a standard curve for the analyte by plotting the analyte concentration on the abscissa (x-axis) and the respective corrected quantitation ratio on the ordinate (y-axis) as shown in Figure 3. Using regression analysis, determine the equation for the curve with respect to the abscissa.

For example, using power regression (13.3.) with the aminopyralid data from Figure 3:

$$Y = \text{constant} \times X^{(\text{exponent})}$$

$$X = \left(\frac{Y}{\text{constant}}\right)^{1/\text{exponent}}$$

$$\text{aminopyralid (µg/mL)} = \left(\frac{\text{aminopyralid quantitation ratio}}{\text{constant}}\right)^{1/\text{exponent}}$$

$$\text{aminopyralid (µg/mL)} = \left(\frac{\text{aminopyralid quantitation ratio}}{102.81}\right)^{1/0.9946}$$

10.3. Calculation of Percent Recovery

10.3.1. For each sample, calculate the uncorrected aminopyralid quantitation ratio.

For example, using the data for aminopyralid from Figure 5c:

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

$$\text{Quantitation Ratio (uncorrected)} = \frac{\text{analyte peak area at } m/z \text{ 263/134}}{\text{ISTD peak area at } m/z \text{ 268/139}}$$

$$\text{Quantitation Ratio (uncorrected)} = \frac{3572}{63559}$$

$$\text{Quantitation Ratio (uncorrected)} = 0.05620$$

10.3.2. For each sample, calculate the corrected aminopyralid quantitation ratio, which accounts for the isotopic contribution of the $^{13}C_2{}^{15}N$- aminopyralid internal standard to the aminopyralid.

For example, using the data for aminopyralid from Figure 4a and 5c:

$$\text{Quantitation Ratio (uncorrected)} = \text{Quantitation Ratio (uncorrected)} - \text{Quantitation Ratio (ISTD)}$$

$$\text{Quantitation Ratio (uncorrected)} = 0.05620 - 0.00184$$

$$\text{Quantitation Ratio (corrected)} = 0.05436$$

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

For example, using the data for aminopyralid from Figure 5:

$$\text{aminopyralid (gross µg/mL)} = \left(\frac{\text{aminopyralid quantitation ratio}}{102.81}\right)^{1/0.9946}$$

$$\text{aminopyralid (gross µg/mL)} = \left(\frac{0.05436}{102.81}\right)^{1/0.9946}$$

$$\text{aminopyralid (gross)} = 0.00051 \text{ µg/mL}$$

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

$$\text{aminopyralid conc. (gross µg/g)} = \text{(µg/mL)} \times \left(\frac{\text{(Extraction vol.) x (Final vol.)}}{\text{(Aliquot x Aliquot factor) x (Sample wt.)}}\right) \text{x Dilution}$$

$$\text{aminopyralid (gross µg/g)} = \text{aminopyralid (µg/mL)} \times \left(\frac{(40\text{ mL}) \times (1.0\text{ mL})}{\left(6.0\text{ mL} \times \frac{1.5\text{ mL}}{3.0\text{ mL}}\right) \times (5.0\text{g})}\right) \times 1$$

$$\text{aminopyralid (gross µg/g)} = 0.00051 \text{ µg/mL} \times \left(\frac{(40\text{ mL}) \times (1.0\text{ mL})}{(6.0\text{ mL} \times 0.5) \times (5.0\text{g})}\right) \times 1$$

$$\text{aminopyralid (gross)} = 0.00136 \text{ µg/g}$$

10.3.5. Determine the net concentration in each recovery sample by subtracting any contribution to the analyte concentration in the control sample from that of the gross analyte concentration in the recovery sample.

For example, using the data for aminopyralid from Figures 5b and 5c:

$$\begin{matrix}\text{aminopyralid} \\ \text{(net µg/g)}\end{matrix} = \begin{matrix}\text{aminopyralid} \\ \text{(gross µg/g)}\end{matrix} - \begin{matrix}\text{aminopyralid} \\ \text{(control µg/g)}\end{matrix}$$

$$\begin{matrix}\text{aminopyralid} \\ \text{(net µg/g)}\end{matrix} = 0.00136\,\mu g/g - 0.0000\,\mu g/g$$

$$\begin{matrix}\text{aminopyralid} \\ \text{(net)}\end{matrix} = 0.00136\ \mu g/g$$

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

$$\text{Recovery} = \frac{\text{Concentration Found}}{\text{Concentration Added}} \times 100\%$$

$$\text{Recovery} = \frac{0.00136\,\mu g/g}{0.0015\,\mu g/g} \times 100\%$$

$$\text{Recovery} = 91\%$$

10.4. Determination of Aminopyralid in Soil

10.4.1. Determine the gross concentration of the analyte in each treated sample by substituting the corrected quantitation ratio obtained into the equation for the standard calibration curve and calculating the uncorrected residue result as described in Sections 10.3.1-10.3.4.

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

For example, using the silt loam data for aminopyralid from Figure 5 and Table 3 for the samples analyzed on 21-Oct-2002:

$$\begin{matrix}\text{aminopyralid} \\ \text{(corrected µg/g)}\end{matrix} = \begin{matrix}\text{aminopyralid} \\ \text{(uncorrected µg/g)}\end{matrix} \times \left(\frac{100}{\%\ \text{Recovery}}\right)$$

$$\begin{matrix}\text{aminopyralid} \\ \text{(corrected µg/g)}\end{matrix} = 0.00136\ \mu g/g \times \frac{100}{88}$$

$$\begin{matrix}\text{aminopyralid} \\ \text{(corrected)}\end{matrix} = 0.00155\ \mu g/g$$

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 dissector containing Drierite adsorbent. Re-weigh the sample when it has cooled to room temperature.

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

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

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

10.6. Determination of Dry Weight Concentrations of Aminopyralid in Soil

10.6.1. Determine the analyte concentration in the sample as described in Sections 10.3.1.-10.3.5.

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

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

$$\text{aminopyralid conc. (dry weight } \mu\text{g/g)} = \text{aminopyralid conc.}(\mu\text{g/g}) \times \left(1 + \frac{\%\ \text{Moisture}}{100}\right)$$

11.4. Standardization of Phenomenex Strata SPE Elution Profile (Note 12.4.)

Variation in the Phenomenex Strata-X SPE 96-well plate may influence the elution profile of the aminopyralid. It is necessary to obtain an elution profile for each lot of SPE plates used to ensure optimum recovery and clean-up efficiency. The following procedure can be used:

11.4.1. Pipet 3.0 mL of 1N hydrochloric acid into a 12-mL culture tube.

11.4.2. Pipet 100 µL of the 0.10 µg/mL aminopyralid fortification solution (Section 7.1.5.) into the culture tube containing the 3.0 mL of hydrochloric acid to obtain a standard solution concentration of 0.003 µg/mL (a final standard solution of 0.005 µg/mL). Vortex mix the solution for approximately 10 seconds.

11.4.3. Place a Phenomenex Strata X SPE 96-well plate on a vacuum manifold equipped for a 96-well plate. Purify the standard solution prepared according to the following SPE procedure:

a. Condition the 96-well plate with 1.0 mL of methanol followed by 1.0 mL of 1N hydrochloric acid (dry plate under full vacuum for 5 seconds between solvents).

b. Transfer 1.5 mL of the standard solution from Step 11.4.2 to the 96 well plate. Pull the sample through the plate at approximately 1 mL/min discarding the eluate. Dry the plate under full vacuum for 5 seconds after the sample has eluted.

c. Wash the plate with 750 µL of a methanol:water (5:95 v/v) solution, discarding the eluate. Dry the cartridge under full vacuum for 15 minutes.

d. Elute the aminopyralid from the plate at approximately 1 mL/min with three 500-µL aliquots of acetonitrile, collecting each 500-µL fraction in a separate 2-mL deep well containing 200 µL of the 0.125-µg/mL aminopyralid stable isotope internal standard solution (Section 7.2.4).

11.4.4. Evaporate the acetonitrile eluate fractions containing internal standard to dryness with nitrogen using a 96-well evaporator set at approximately 50 °C with a flow rate of approximately 500 mL/minute.

11.4.5. Reconstitute the eluted fractions in 200 µL of the acetonitrile/pyridine/butanol solution (22:2:1), and vortex mix the samples for approximately 1 minute.

11.4.6. Add 200-µL aliquots of the calibration standards and crossover standards in the acetonitrile/pyridine/butanol solution (22:2:1) to individual empty wells in the collection plate.

11.4.7. Derivatize the eluted fractions and standards by adding 10 µL of butyl chloroformate to each well.

11.4.8. Allow the eluted fractions and standards to stand at room temperature for approximately 5 minutes to ensure complete derivatization.

11.4.9. Add 790 µL of the methanol/water/acetic acid (50:50:0.1) solution to each eluted fraction and standard.

11.4.10. Cap the 96-well collection plate and vortex mix gently for approximately 30 seconds.

11.4.11. Analyze the eluted fractions along with the derivatized calibration standards using the LC/MS/MS conditions described in Section 8.

11.4.12. Calculate the percent recovery according to the procedure outlined in Section 10.2. and Section 10.3.

If the elution profile differs from that suggested in the method and shown in Figure 6, adjust the volume of acetonitrile to be used for elution in Step 9.3.19.e of the sample analysis.

12. NOTES

12.1. Equipment, glassware, materials, reagents, and chemicals considered to be equivalent to those specified may be substituted with the understanding that their performance must be confirmed by appropriate tests. Common laboratory supplies are assumed to be readily available and are, therefore, not listed.

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

12.3. Operating conditions may be modified to obtain optimal chromatographic separation and performance, if necessary.

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

13. REFERENCES

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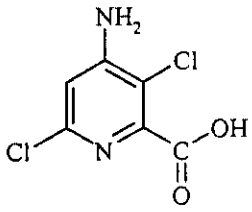
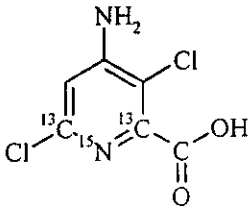
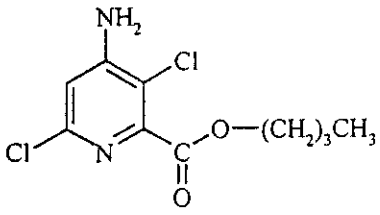
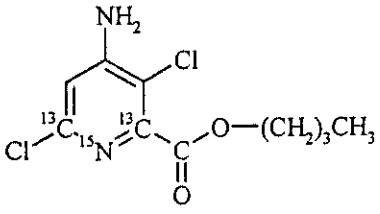
Table 1. Identity and Structures of Aminopyralid and Related Compounds

Common Name of Compound	Structure and CAS Name
Aminopyralid Molecular Formula: $C_6H_4Cl_2N_2O_2$ Nominal Mass: 206 CAS Number: 150114-71-9	 4-amino-3,6-dichloro-2-pyridinecarboxylic acid
$^{13}C_2{}^{15}N$-Aminopyralid Molecular Formula: $^{13}C_2C_4H_4Cl_2{}^{15}NNO_2$ Nominal Mass: 209 CAS Number: not available	 4-amino-3,6-dichloro-2-[$^{13}C_2{}^{15}N$]pyridinecarboxylic acid
Aminopyralid 1-butyl ester Molecular Formula: $C_{10}H_{12}Cl_2N_2O_2$ Nominal Mass: 262 CAS Number: not available	 4-amino-3,6-dichloro-2-pyridinecarboxylic acid, 1-butyl ester
$^{13}C_2{}^{15}N$-Aminopyralid 1-butyl ester Molecular Formula: $^{13}C_2C_8H_{12}Cl_2{}^{15}NNO_2$ Nominal Mass: 265 CAS Number: not available	 4-amino-3,6-dichloro-2-[$^{13}C_2{}^{15}N$]pyridine-carboxylic acid, 1-butyl ester