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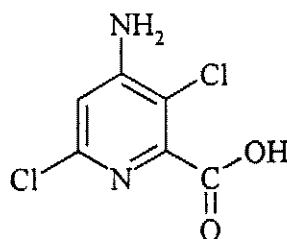


Determination of Residues of Aminopyralid in Water by Liquid Chromatography with Tandem Mass Spectrometry Detection

M. J. Hastings

1. SCOPE

This method is applicable for the quantitative determination of residues of aminopyralid (4-amino-3,6-dichloro-2-pyridinecarboxylic acid) in water (drinking water, ground water and surface water). The method was validated over the concentration range of 0.05-5.0 µg/L. The validated limit of quantitation for the method is 0.05 µg/L.



Aminopyralid
CAS 150114-71-9

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

2. PRINCIPLE

Residues of aminopyralid are extracted from an acidified water sample using a polymeric solid phase extraction cartridge (SPE). The SPE cartridge is washed with a water:methanol:formic acid solution (90:10:1) and eluted with a methyl-*tert*-butyl ether:methanol solution (90:10). An aliquot of an aminopyralid stable isotope solution is added to the sample and the eluate is evaporated to dryness. The residue is reconstituted in an acetonitrile:pyridine:butanol (22:2:1) solution and the sample and standards are derivatized with butyl chloroformate. The sample and standards are then diluted with a methanol:water:acetic acid mobile phase (50:50:0.1) and 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 0.9997. 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 the 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, methanol and methyl-*tert*-butyl ether are flammable and volatile and should be used in well-ventilated areas away from ignition sources.
- 3.3. Acetic acid, formic acid and sulfuric 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 is highly toxic. It is imperative that proper eye and personal protection equipment be used when handling all chemicals.
- 3.5. Pyridine is a possible carcinogen. Pyridine is also flammable and volatile and should be used in well-ventilated areas away from ignition sources. 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., Hightstown, NJ 08520.
- 4.1.2. Evaporator, TurboVap LV, Zymark Corporation, Hopkinton, MA 01748.
- 4.1.3. Pipetter, adjustable, Eppendorf, 5-100 μ L, catalog number 21-378-74, Fisher Scientific, Pittsburgh, PA 15275.
- 4.1.4. Pipetter, adjustable, Eppendorf, 20-300 μ L, catalog number 21-378-77, Fisher Scientific.

- 4.1.5. Pipetter, adjustable, Eppendorf, 50-1000 μ L, catalog number 21-378-83, Fisher Scientific.
- 4.1.6. Pipetter, adjustable, Eppendorf, 1000 - 5000 μ L, catalog number 21-378-79, Fisher Scientific.
- 4.1.7. SPE reservoir, 10-mL, catalog number 120-1005-H, International Sorbent Technology Ltd, Hengoed, Mid Glamorgan UK and distributed by Argonaut Technologies, Foster City, CA 94404.
- 4.1.8. Vacuum manifold, VacMaster-20, catalog number 121-2027, International Sorbent Technology Ltd, Hengoed, Mid Glamorgan UK and distributed by Argonaut Technologies.
- 4.1.9. 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- μ m, catalog number 030C18Q-10046, Diazem Corporation, Midland, MI 48640.
 - 4.2.2. Liquid Chromatograph autosampler, Model 1100, Agilent Technologies. Agilent Technologies, Palo Alto, CA 94303.
 - 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. Bottle, 250-mL, media bottle, catalog number 06-423-3B, Fisher Scientific.
 - 5.2. Bottle, 500-mL, media bottle, catalog number 06-423-3C, Fisher Scientific.
 - 5.3. Bottle, 1-L, media bottle, catalog number 06-423-3D, Fisher Scientific.
 - 5.4. Cylinder, graduated mixing, 50-mL, catalog number 08-565C, Fisher Scientific.
 - 5.5. Cylinder, graduated mixing, 250-mL, catalog number C7000-250, National Scientific Company.
 - 5.6. Cylinder, graduated mixing, 500-mL, catalog number C7000-500, National Scientific Company.

- 5.7. Cylinder, graduated mixing, 1-L, catalog number C7000-1L, National Scientific Company.
- 5.8. Flask, volumetric, 100-mL, catalog number 10-206D, Fisher Scientific.
- 5.9. Flask, volumetric, 200-mL, catalog number 10-206E, Fisher Scientific.
- 5.10. Flask, volumetric, 500-mL, catalog number 10-206G, Fisher Scientific.
- 5.11. Pipet, 3.2-mL disposable transfer, catalog number 13-711-7, Fisher Scientific.
- 5.12. Pipet, volumetric, 0.5-mL, catalog number 261-6010, National Scientific Company.
- 5.13. Pipet, volumetric, 1.0-mL, catalog number 261-6011, National Scientific Company.
- 5.14. Pipet, volumetric, 2.0-mL, catalog number 261-6012, National Scientific Company.
- 5.15. Pipet, volumetric, 3.0-mL, catalog number 261-6013, National Scientific Company.
- 5.16. Pipet, volumetric, 5.0-mL, catalog number 261-6015, National Scientific Company.
- 5.17. Pipet, volumetric, 10.0-mL, catalog number 261-6020, National Scientific Company.
- 5.18. Pipetter tips, Brinkmann Eppendorf, 1-200- μ L tip, catalog number 21-371-3, Fisher Scientific.
- 5.19. Pipetter tips, Brinkmann Eppendorf, 1000- μ L tip, catalog number 22350901, Brinkmann Instruments, Inc.
- 5.20. Pipetter tips, Brinkmann Eppendorf, 5-mL tip, catalog number 22350811, Brinkmann Instruments, Inc.
- 5.21. SPE cartridges, Phenomenex Strata X 33 μ m, 3-mL, 60-mg packing, catalog number 8E-S100-UBJ, Phenomenex, Torrance, CA 90501.
- 5.22. Tubes, culture, 12-mL (16 x 100 mm), catalog number 99449-16, Corning Products, Corning, NY 14831.
- 5.23. 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. Formic acid, 96%, ACS Grade, catalog number, 25,136-4, Sigma-Aldrich.
- 6.1.6. Methanol, HPLC grade, catalog number 3041, Mallinckrodt Baker, Inc.
- 6.1.7. Methyl *tert*-butyl ether (MTBE), OmniSolv grade, catalog number MX0826-1, EM Science, Gibbstown, NJ 08027.
- 6.1.8. Nitrogen, refrigerated liquid, catalog number LQNI, BOC Gases, New Providence, NJ 07974.
- 6.1.9. Pyridine, ACS reagent grade, catalog number 18, 452-7, Sigma-Aldrich.
- 6.1.10. Sulfuric acid, Concentrated, certified ACS Plus, catalog number A300-500, Fisher Scientific.
- 6.1.11. Water, HPLC grade, catalog number WX0004-1, Fisher Scientific.

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.
- 6.2.2. Aminopyralid-1-15N-2,6-13C: 4-amino-3,6-dichloro-2-pyridinecarboxylic acid
Obtain from Specialty Synthesis COE, 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/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. Transfer the solution to a 1-L solvent bottle. Allow the solution to equilibrate to room temperature before use.

6.3.2. methanol/acetic acid (100:0.1)

Pipette 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 solvent bottle.

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

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

6.3.4. methyl *tert*-butyl ether/methanol (90:10)

Combine 180 mL of methyl *tert*-butyl ether and 20 mL of methanol in a 250-mL graduated mixing cylinder. Place a glass stopper on the cylinder and invert several times to mix. Transfer the solution to a 250-mL solvent bottle. 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 HPLC water. Place a glass stopper on the cylinder and invert several times to mix. Transfer the solution to a 1-L solvent bottle.

6.3.6. water/methanol (90:10)

Combine 450 mL of HPLC water and 50 mL of methanol in a 500-mL graduated mixing cylinder. Place a glass stopper on the cylinder and invert several times to mix. Transfer the solution to a 500-mL solvent bottle. Allow the solution to equilibrate to room temperature before use.

6.3.7. water/methanol/formic acid (90:10:1)

Combine 180 mL of HPLC grade water and 20 mL of methanol in a 250-mL graduated mixing cylinder. Pipet 2.0 mL of formic acid into the solvent mixture. Place a glass stopper on the cylinder and invert several times to mix. Transfer the solution to a 250-mL solvent bottle. Allow the solution to equilibrate to room temperature before use.

6.3.8. water/conc. sulfuric acid (99.50:0.5)

Pipette 1.0 mL of conc. sulfuric acid into a 250-mL graduated mixing cylinder. Carefully add 200 mL of HPLC water. Place a glass stopper on the cylinder and invert

several times to mix. Transfer the solution to a 250-mL solvent bottle. Allow the solution to equilibrate to room temperature before use.

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- $\mu\text{g}/\text{mL}$ stock solution.
- 7.1.2. Pipet 10.0 mL of the 1000- $\mu\text{g}/\text{mL}$ solutions in Section 7.1.1. into a 100-mL volumetric flask and dilute to volume with acetonitrile to obtain a 100.0- $\mu\text{g}/\text{mL}$ fortification solution.
- 7.1.3. Pipet 1.0 mL of the 100.0- $\mu\text{g}/\text{mL}$ standard solution in Section 7.1.2. into a 100-mL volumetric flask and dilute to volume with a water/methanol solution (90:10) to obtain a 1.0- $\mu\text{g}/\text{mL}$ fortification solution.
- 7.1.4. Pipet 10.0 mL of the 1.0- $\mu\text{g}/\text{mL}$ fortification solution in Section 7.1.3. into a 100-mL volumetric flask and dilute to volume with a water/methanol solution (90:10) to obtain a 0.1- $\mu\text{g}/\text{mL}$ fortification solution.
- 7.1.5. Pipet 10.0 mL of the 0.1- $\mu\text{g}/\text{mL}$ fortification solution in Section 7.1.4. into a 100-mL volumetric flask and dilute to volume with a water/methanol solution (90:10) to obtain a 0.01- $\mu\text{g}/\text{mL}$ 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. Dilute to volume with an acetonitrile:pyridine:butanol (22:2:1) solution to obtain a nominal 100.0- $\mu\text{g}/\text{mL}$ stock solution.
- 7.2.2. Pipet 5.0 mL of the nominal 100.0- $\mu\text{g}/\text{mL}$ 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- $\mu\text{g}/\text{mL}$ internal standard solution.
- 7.2.3. Pipet 1.0 mL of the nominal 100.0- $\mu\text{g}/\text{mL}$ stable isotope stock solution in Section 7.2.1. into a 100-mL volumetric flask and dilute to volume with acetonitrile to obtain a nominal 1.0- $\mu\text{g}/\text{mL}$ internal standard solution.
- 7.2.4. Pipet 25.0 mL of the nominal 1.0- $\mu\text{g}/\text{mL}$ stable isotope internal standard solution in Section 7.2.3. into a 200-mL volumetric flask and dilute to volume with acetonitrile to obtain a nominal 0.125- $\mu\text{g}/\text{mL}$ internal standard solution.

7.3. Preparation of Aminopyralid Calibration Solutions

- 7.3.1. Pipet 1.0 mL of the 100.0- $\mu\text{g/mL}$ aminopyralid fortification solution from Section 7.1.2. into a 100-mL volumetric flask and dilute to volume with an acetonitrile:pyridine:butanol (22:2:1) solution to obtain a 1.0- $\mu\text{g/mL}$ calibration standard stock solution.
- 7.3.2. Pipet 10.0 mL of the 1.0- $\mu\text{g/mL}$ calibration solution from Section 7.3.1. into a 100-mL volumetric flask and dilute to volume with an acetonitrile:pyridine:butanol (22:2:1) solution to obtain a 0.1- $\mu\text{g/mL}$ calibration solution.
- 7.3.3. Pipet 10.0 mL of the 0.1- $\mu\text{g/mL}$ calibration solution from Section 7.3.2. into a 100-mL volumetric flask and dilute to volume with an acetonitrile:pyridine:butanol (22:2:1) solution to obtain a 0.01- $\mu\text{g/mL}$ calibration solution.
- 7.3.4. Prepare calibration solutions by pipetting 12.5 mL of the nominal 1.0- $\mu\text{g/mL}$ stable isotope internal standard solution (Section 7.2.2.) into each 100-mL volumetric flask and then diluting the calibration standard stock solutions from Section 7.3.1.-7.3.3. with an acetonitrile:pyridine:butanol (22:2:1) solution as described in the table overleaf:

Original Standard Concentration	Aliquot of Original Standard	Final Solution Volume	Calib. Soln. Final Concentration	Calib. Soln. Concentration Post Derivatization ^a	Equivalent Sample Concentration
$\mu\text{g/mL}$	mL	mL	$\mu\text{g/mL}$	$\mu\text{g/mL}$	$\mu\text{g/L}$
1.0	25.0	100.0	0.25	0.05	5.0
1.0	17.5	100.0	0.175	0.035	3.5
1.0	10.0	100.0	0.10	0.02	2.0
1.0	5.0	100.0	0.05	0.01	1.0
1.0	2.5	100.0	0.025	0.005	0.50
0.10	5.0	100.0	0.005	0.001	0.10
0.10	2.5	100.0	0.0025	0.0005	0.05
0.01	5.0	100.0	0.0005	0.0001	0.01

^a Equivalent sample concentration after derivatizing 200 μL of each calibration standard and diluting to a final volume of 1.0 mL.

7.4. Preparation of Aminopyralid and Aminopyralid Stable Isotope Cross Over Standard Solutions

- 7.4.1. Prepare an aminopyralid cross over standard solution by pipetting 12.5 mL of the 1.0- $\mu\text{g/mL}$ aminopyralid stock calibration solution (Section 7.3.1.) into a 100-mL volumetric flask and diluting to volume with an acetonitrile:pyridine:butanol (22:2:1) solution to produce a 0.125- $\mu\text{g/mL}$ aminopyralid cross over standard solution.
- 7.4.2. Prepare an aminopyralid stable isotope cross over standard solution by pipetting 12.5 mL of the 1.0- $\mu\text{g/mL}$ 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 to produce a 0.125- μ g/mL aminopyralid stable isotope cross over standard 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- μ m

Column Temperature: 35 °C

Injection Volume: 50 μ L
Run Time: 6.0 minutes

Mobile Phase: A –Methanol with 0.1% acetic acid
B –Water with 0.1% acetic acid

Flow Rate: 900 μ L/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
Collision Gas (CAD): 12
Temperature (TEM): 425 °C
Ion Source Gas 1 (GS1): 8
Ion Source Gas 2 (GS2): 7000

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

	<u>Ion, m/z</u>		Time, ms	Collision Energy, v
	Q1	Q3		
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 water is presented in Figure 3.

8.5. Typical Chromatograms

Typical chromatograms of a 0.025- $\mu\text{g}/\text{mL}$ aminopyralid butyl ester cross over standard and a 0.025- $\mu\text{g}/\text{mL}$ aminopyralid stable isotope butyl ester cross over standard are presented in Figure 4.

Typical chromatograms of a 0.0005- $\mu\text{g}/\text{mL}$ calibration standard, a control tap water sample, a control tap water sample fortified at 0.05 $\mu\text{g}/\text{L}$ (limit of quantitation), and a control tap water sample fortified at 0.5 $\mu\text{g}/\text{L}$ (10 times the limit of quantitation) are presented in Figure 5.

9. DETERMINATION OF RECOVERY OF AMINOPYRALID FROM WATER

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

No sample preparation is required for water samples. Samples should be stored refrigerated or deep frozen prior to analysis.

9.3. Sample Analysis

- 9.3.1. Pipet a 10.0-mL aliquot of the water sample into a 40-mL vial.
- 9.3.2. For recovery samples, add 50- μ L aliquots of the 0.01, 0.1, and 1.0- μ g/mL fortification solutions (Sections 7.1.3. – 7.1.5.) to control water samples to obtain concentrations ranging from 0.05 to 5.0 μ g/L.
- 9.3.3. Carefully add 50 μ L of concentrated sulfuric acid to the sample vial. Cap the vial and mix.
- 9.3.4. Concentrate and purify the sample using the following SPE procedure (Note 12.4.):
- Attach a 10-mL reservoir to a 60-mg (3-mL) Phenomenex Strata X SPE cartridge.
 - Condition the SPE cartridge with 3 mL of methanol followed by 3 ml of water/concentrated sulfuric acid (99.5:0.5). Dry the cartridge under full vacuum for 5 seconds between solvents.
 - Transfer the acidified water sample from Step 9.3.3. to the SPE cartridge. Pull the water through the cartridge at approximately 2 mL/min, discarding the eluate. Dry the cartridge under full vacuum for 30 seconds.
 - Rinse the 40-mL vial with 2 mL of water/methanol/formic acid (90:10:1) and apply the rinse to the SPE cartridge.
 - Air dry the SPE cartridge for 20 minutes at full vacuum (approx. 20 inches of mercury).
 - Elute the aminopyralid from the SPE cartridge at approximately 1 mL/min with two 2.0-mL aliquots of methyl *tert*-butyl ether/methanol (90:10), collecting the eluate in 12-mL culture tubes.
- 9.3.5. Pipet 200 μ L of the 0.125- μ g/mL stable isotope standard solution (Section 7.2.4.) into the 12-mL culture tube containing the elution solvent.
- 9.3.6. Evaporate the elution solvent to dryness in a TurboVap evaporator set at 40 °C (10 psi nitrogen gas flow).
- 9.3.7. Pipet 200 μ L of the 0.125 μ g/mL aminopyralid cross over standard (Section 7.4.1.) into a new culture tube to produce the analyte→ISTD cross over standard.

- 9.3.8. Pipet 200 μL of the 0.125 $\mu\text{g}/\text{mL}$ aminopyralid stable isotope standard solution (Section 7.4.2.) into a new culture tube to produce the ISTD→analyte cross over standard.
- 9.3.9. Pipet 200- μL of each calibration standard (Section 7.3.4.) into a series of new 12-mL culture tubes.
- 9.3.10. Pipet 200 μL of the acetonitrile/pyridine/1-butanol (22:2:1) into the sample vial from step 9.3.6 and vortex mix for approximately 10 seconds.
- 9.3.11. Pipet 10 μL of butyl chloroformate into the cross over standard vials (Section 9.3.7 and 9.3.8.), the standards vials (Section 9.3.9) and the sample vial (Section 9.3.10).
- 9.3.12. Vortex mix for 5 seconds, ensuring that the liquid coats the sides of the vial.
- 9.3.13. Allow the sample and standards to derivatize for approximately 5 minutes at ambient temperature.
- 9.3.14. Pipet 790 μL of the methanol:water:acetic acid solution (50:50:0.1) into each standard and sample vial. Vortex mix the samples and standards for approximately 10 seconds.
- 9.3.15. Analyze the samples and the calibration standards using LC/MS/MS conditions listed in Section 8. Determine the suitability of the chromatographic system using the following criteria:
- 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.
 - 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 0.0005- $\mu\text{g}/\text{mL}$ calibration standard (equivalent to 0.05 $\mu\text{g}/\text{L}$ of aminopyralid in the water sample).
- 9.3.16. If the sample concentrations exceed the range of the standard calibration curve, dilute the sample with derivatized $^{13}\text{C}_2$ ^{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 $^{13}\text{C}_2^{15}\text{N}$ -aminopyralid, inject the crossover standards described in Sections 7.4.1 and 7.4.2 and determine the peak areas for the analyte and internal standard as indicated below.

aminopyralid	m/z Q1/Q3 263/134
$^{13}\text{C}_2^{15}\text{N}$ -aminopyralid	m/z Q1/Q3 268/139

For example, to determine the contribution of the unlabeled aminopyralid to the labeled $^{13}\text{C}_2^{15}\text{N}$ -aminopyralid internal standard using the aminopyralid data from Figure 4a:

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

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

$$\text{Crossover Factor (analyte} \rightarrow \text{ISTD)} = \frac{2699.6}{681203.5} = 0.0040$$

In a similar manner, to determine the contribution of the labeled $^{13}\text{C}_2^{15}\text{N}$ -aminopyralid internal standard to the unlabeled aminopyralid using the $^{13}\text{C}_2^{15}\text{N}$ -aminopyralid data from Figure 4b:

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

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

$$\text{Crossover Factor (ISTD} \rightarrow \text{analyte)} = \frac{710.8}{223184.6} = 0.0032$$

The average crossover factor calculated from data generated in the validation study was 0.0028.

The crossover factors illustrated above indicate that the (analyte \rightarrow ISTD) crossover is greater than the (ISTD \rightarrow analyte) crossover. However, the amount of the $^{13}\text{C}_2^{15}\text{N}$ -aminopyralid internal standard used in the method was chosen to minimize the effect of the (analyte \rightarrow 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 \rightarrow 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. Determine the peak areas for the analyte and internal standard as indicated below.

aminopyralid *m/z* Q1/Q3 263/134
¹³C₂¹⁵N- aminopyralid *m/z* Q1/Q3 268/139

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

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

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

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

$$\text{Quantitation Ratio (uncorrected)} = \frac{8466.3}{135116.9}$$

$$\text{Quantitation Ratio (uncorrected)} = 0.06266$$

10.2.3. For each standard, calculate the corrected aminopyralid quantitation ratio, which accounts for the isotopic contribution of the ¹³C₂¹⁵N-aminopyralid internal standard to the aminopyralid.

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

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

$$\text{Quantitation Ratio (corrected)} = 0.06266 - 0.0028$$

$$\text{Quantitation Ratio (corrected)} = 0.05986$$

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:

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

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

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

$$\text{aminopyralid} \text{ (}\mu\text{g/mL)} = \left(\frac{\text{aminopyralid quantitation ratio}}{136.2909} \right)^{1/1.0174}$$

10.3. Calculation of Percent Recovery

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

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

$$\text{aminopyralid} \text{ (gross } \mu\text{g/mL)} = \left(\frac{\text{aminopyralid quantitation ratio}}{136.2909} \right)^{1/1.0174}$$

$$\text{aminopyralid} \text{ (gross } \mu\text{g/mL)} = \left(\frac{0.0620}{136.2909} \right)^{1/1.0174}$$

$$\text{aminopyralid} \text{ (gross)} = 0.00052 \mu\text{g/mL}$$

- 10.3.2. Convert the concentration ($\mu\text{g/mL}$) of aminopyralid found in the sample solution prepared for analysis to the concentration ($\mu\text{g/L}$) of aminopyralid found in the original sample as follows:

$$\text{aminopyralid} \text{ (gross } \mu\text{g/L)} = \text{aminopyralid} \text{ (}\mu\text{g/mL)} \times \left(\frac{1000 \text{ mL}}{10.0 \text{ mL}} \times 1.0 \text{ mL} \right)$$

$$\text{aminopyralid} \text{ (gross)} = 0.052 \mu\text{g/L}$$

- 10.3.3. Determine the net concentration in each recovery sample by subtracting any contribution resulting from 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:

$$\begin{array}{l} \text{aminopyralid} \\ \text{(net } \mu\text{g/L)} \end{array} = \begin{array}{l} \text{aminopyralid} \\ \text{(gross } \mu\text{g/L)} \end{array} - \begin{array}{l} \text{aminopyralid} \\ \text{(control } \mu\text{g/L)} \end{array}$$

$$\begin{array}{l} \text{aminopyralid} \\ \text{(net } \mu\text{g/L)} \end{array} = 0.052 \mu\text{g/L} - 0.002 \mu\text{g/L}$$

$$\begin{array}{l} \text{aminopyralid} \\ \text{(net)} \end{array} = 0.05 \mu\text{g/L}$$

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

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

$$\text{Recovery} = \frac{0.05 \mu\text{g/L}}{0.05 \mu\text{g/L}} \times 100\%$$

$$\text{Recovery} = 100\%$$

10.4. Determination of Aminopyralid in Water

- 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 10.3.1-10.3.2.
- 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 tap water data for aminopyralid from Figure 5 and Table 2 for the samples analyzed on 11-Feb-2003:

$$\begin{array}{l} \text{aminopyralid} \\ \text{(corrected } \mu\text{g/L)} \end{array} = \begin{array}{l} \text{aminopyralid} \\ \text{(uncorrected } \mu\text{g/L)} \end{array} \times \left(\frac{100}{\% \text{ Recovery}} \right)$$

$$\begin{aligned} \text{aminopyralid} &= 0.05 \mu\text{g/L} \times \frac{100}{100} \\ \text{(corrected } \mu\text{g/L)} & \end{aligned}$$

$$\begin{aligned} \text{aminopyralid} &= 0.05 \mu\text{g/g} \\ \text{(corrected)} & \end{aligned}$$

11.4. Standardization of Phenomenex Strata SPE Elution Profile

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

- 11.4.1. Pipet a 10-mL aliquot of HPLC grade water into a 40-mL vial.
- 11.4.2. Pipet 50 μ L of the 1.0 μ g/mL fortification solution (Section 7.1.3.) into the HPLC grade water.
- 11.4.3. Carefully add 50 μ L of concentrated sulfuric acid to the sample vial. Cap the vial and mix.
- 11.4.4. Profile the SPE cartridge using the following procedure:
 - a) Attach a 10 mL reservoir to a 60-mg (3-mL) Phenomenex Strata X SPE cartridge.
 - b) Condition the SPE cartridge with 3 mL of methanol followed by 3 ml of water/concentrated sulfuric acid (99.5:0.5). Dry the cartridge under full vacuum for 5 seconds between solvents.
 - c) Transfer the acidified water sample from Step 11.4.3. to the SPE cartridge. Pull the water through the cartridge at approximately 2 mL/min, discarding the eluate. Dry the cartridge under full vacuum for 30 seconds.
 - d) Rinse the 40-mL vial with 2 mL of water/methanol/formic acid (90:10:1) and apply the rinse to the SPE cartridge.
 - e) Air dry the SPE cartridge for 20 minutes at full vacuum (20 inches of mercury).
 - f) Elute the aminopyralid from the SPE cartridge at approximately 1 mL/min with three 2.0-mL aliquots of methyl *tert*-butyl ether/methanol (90:10) collecting the eluate in separate 12-mL culture tubes.
- 11.4.5. Pipet 200 μ L of the 0.125- μ g/mL stable isotope standard solution (Section 7.2.4.) into the 12-mL culture tubes containing the elution solvent.
- 11.4.6. Evaporate the elution solvent to dryness in a TurboVap evaporator set at 40 °C (10 psi nitrogen gas flow).
- 11.4.7. Pipet 200- μ L of each calibration standard (Section 7.3.4.) into clean 12-mL culture tubes.

- 11.4.8. Pipet 200 μ L of the butylation coupling reagent (acetonitrile:pyridine:1-butanol 22:2:1) into the sample vials from step 11.4.6. and vortex mix for approximately 10 seconds.
- 11.4.9. Pipet 10 μ L of butyl chloroformate into the standard and sample vials (Section 11.4.7. and 11.4.8.). Vortex mix for 5 seconds, ensuring the liquid coats the sides of the vial.
- 11.4.10. Allow the samples and standards to derivatize for approximately 5 minutes at ambient temperature.
- 11.4.11. Pipet 790 μ L of the methanol:water:acetic acid solution (50:50:0.1) into each standard and sample vial. Vortex mix the samples and standards for approximately 10 seconds.
- 11.4.12. Analyze the elution fractions and the calibration standards using LC/MS/MS conditions listed in Section 8.
- 11.4.13. Calculate the percent recovery according to the procedure outlined in Section 10.3. If the elution profile differs from that illustrated in Figure 6, adjust the volume of methyl *tert*-butyl ether/methanol (90:10) to be used for elution in Step 9.3.4.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. 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 cartridges, determine the elution profile as described in Section 11.4.

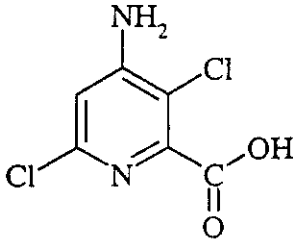
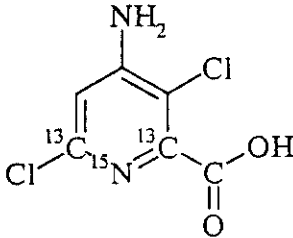
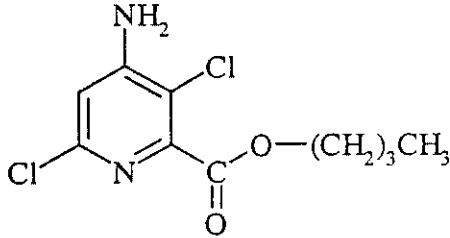
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Table 1. Identity and Structures of Aminopyralid and Aminopyralid- $1-^{15}N-2,6-^{13}C$

Common Name of Compound	Structure
aminopyralid Molecular Formula: $C_6H_4Cl_2N_2O_2$ Nominal Mass: 206	aminopyralid 
aminopyralid- $1-^{15}N-2,6-^{13}C$ Molecular Formula: $^{13}C_2C_4H_4Cl_2^{15}NNO_2$ Nominal Mass: 209	aminopyralid- $1-^{15}N-2,6-^{13}C$ 
aminopyralid butyl ester Molecular Formula: $C_{10}H_{12}Cl_2N_2O_2$ Nominal Mass: 262	aminopyralid butyl ester 
aminopyralid butyl ester- $1-^{15}N-2,6-^{13}C$ Molecular Formula: $^{13}C_2C_8H_{12}Cl_2^{15}NNO_2$ Nominal Mass: 265	aminopyralid butyl ester- $1-^{15}N-2,6-^{13}C$ 