STUDY TITLE

Independent Laboratory Validation of Aminopyralid in Compost

DATA REQUIREMENTS

SANCO/825/00 Rev. 8.1 (2010); U.S. EPA Guidance OPPTS 860.1000, Background, OCSPP 850.6100, Independent Laboratory Validation

Independent Laboratory Validation of Aminopyralid in Compost

INTRODUCTION

The purpose of this study was to demonstrate that analytical method "Method Validation Study for the Determination of Residues of Aminopyralid in Compost Using Liquid Chromatography with Tandem Mass Spectrometry", ID 191576, could be performed successfully at an outside facility with no prior experience with the method (Reference 1).

Principle of the method. Residues of aminopyralid are extracted from the sample matrices by shaking with 0.1N NaOH and then centrifuged. An aliquot of the extract is transferred. The sample is treated with an aliquot of concentrated (12.1 N) HCl and heated for 90 min at 90°C to hydrolyze any bound aminopyralid in the starting material. Samples are centrifuged to enable removal of some denser materials, and then undergo solid phase extraction. After internal standard (IS) is added, samples are evaporated to dryness, derivatized, and then reconstituted for analysis. Solvent calibration standards spiked with IS are also derivatized. Analysis occurs via liquid chromatography with positive-ion electrospray ionization tandem mass spectrometry (LC-MS/MS).

Test conditions. For validation, the analytical set consisted of two reagent blanks, two matrix controls, one control fortified at LOD (limit of detection), five replicates fortified at LOQ (limit of quantitation), five replicates fortified at 10X LOQ, and five replicated fortified at 80X LOQ. The mass transitions used for analysis are listed below.

	Quantitation (*m/z*)
Aminopyralid	*m/z* 262.9 → 133.9
Aminopyralid[1]	*m/z* 264.9 → 135.9
Aminopyralid IS	*m/z* 269.0 → 111.0

[1]Denotes confirmatory ion.

Limit of Quantification (LOQ) and Limit of Detection (LOD). During the independent laboratory validation of the method, the limit of quantitation (LOQ) of aminopyralid was confirmed to be 0.5 ng/g (ng/g) for compost. The LOD for aminopyralid was set at 30% of the defined LOQ.

Selectivity. At the retention time of aminopyralid and aminopyralid IS, no interfering peaks were found.

Standard Stability. Analytical standards and fortification solutions were stored under refrigerated conditions when not in use. Stock standard and spiking standard stabilities were assessed in a separate study, and solutions of aminopyralid were found to be stable in acetonitrile for at least 198 days

Extract Stability. Extract stability was established during validation study "Method Validation Study for the Determination of Residues of Aminopyralid in Compost Using Liquid Chromatography with Tandem Mass Spectrometry", ID 191576 (Reference 1). Sample extracts from pasture compost and manure compost were tested after 4 days of storage at refrigerated temperature and were determined to be stable.

Recovery and Repeatability. The independent laboratory validation (ILV) was performed successfully. Apparent residues of aminopyralid were below the method limit of detection (0.15 ng/g) in all of the control samples.

Conclusion. The results of this independent laboratory validation (ILV) study demonstrate that analytical method "Method Validation Study for the Determination of Residues of Aminopyralid in Compost Using Liquid Chromatography with Tandem Mass Spectrometry", ID 191576, fulfils the requirements with regard to specificity, repeatability, limit of quantification, and recoveries for compost.

EXPERIMENTAL

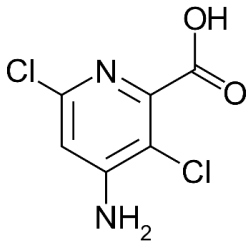
Test Systems

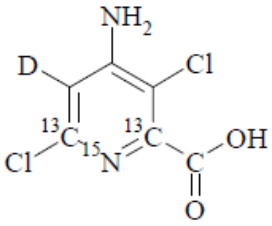
The test system considered in this study was manure compost. The control sample was provided by Dow AgroSciences and sent to SGS via overnight shipping on dry ice. The compost sample was received by SGS on November 12, 2019. The test system was received frozen and stored under frozen conditions at all times, unless necessary for laboratory analysis.

Test and Reference Substances

The aminopyralid standard was stored at room temperature. The aminopyralid internal standard was stored in the refrigerator. The sponsor has retained a reserve sample of these chemicals, and has documentation specifying the location of the synthesis and characterization information available at Dow AgroSciences in Indianapolis, Indiana.

The aminopyralid and aminopyralid internal standard reference substances were provided by the sponsor and received on October 29, 2019. Upon receipt, the standard substances were stored in the ambient and refrigerated lockboxes with the temperature ranging from 20 to 23 °C and 1 to 8 °C, respectively. All standards were stored under locked conditions. The certificates of analysis are presented in Appendix A. A detailed summary of the reference substances is presented below.

Common Name	Aminopyralid
Chemical Formula	$C_6H_4Cl_2N_2O_2$
Test Substance Structure	
CAS Number	150114-71-9
Supplier	Dow AgroSciences
Lot / Batch #	YC2-142695-9
Purity	99%
Expiration	June 24, 2022

Common Name	Aminopyralid M+4
Chemical Formula	$C_4{}^{13}C_2H_3DCl_2N^{15}NO_2$
Test Substance Structure	
CAS Number	N/A
Supplier	Dow AgroSciences
Lot / Batch #	DE3-169801-5
Purity	N/A
Expiration	Sep 08, 2022

Analytical Method

Analytical method "Method Validation Study for the Determination of Residues of Aminopyralid in Compost Using Liquid Chromatography with Tandem Mass Spectrometry", ID 191576, was used for the analysis of the samples.

Residues of aminopyralid were extracted from compost by shaking with 0.1N NaOH. An aliquot of the extract was transferred and treated with an aliquot of concentrated (12.1 N) HCl and heated for 90 min at 90°C to hydrolyze any bound aminopyralid in the starting material. Samples were centrifuged to remove some denser materials, and then underwent solid phase extraction. After internal standard (IS) was added, samples were evaporated to dryness, derivatized, and then reconstituted for analysis. Solvent calibration standards spiked with IS were also derivatized. Analysis occurred via liquid chromatography with positive ion electrospray ionization tandem mass spectrometry (LC-MS/MS). The primary (quantitative) and secondary (confirmatory) transition ions monitored are presented on the following page:

Analyte	Transition (*m/z*)		Ionization Mode	Retention Time (min)
	Primary	Secondary		
Aminopyralid	262.9 → 133.9	264.9 → 135.9	Positive ESI	~1.9
Aminopyralid IS	269.0 → 111.0	N/A		~1.9

Matrix Effect Testing

Matrix effects were evaluated at 10x LOQ (5.0 ng/g) by comparing the peak area response of aminopyralid fortified in a control extract after processing to the peak area response of aminopyralid fortified in neat solvent.

However, as suggested in the method validation, the aminopyralid stable isotope was used in order to normalized these observed matrix effects. Therefore, quantitation was achieved with the aid of the stable isotope internal standard and measuring peak area response ratio.

This value is based solely on the response of aminopyralid and does not account for the compensation to the matrix effect provided by the aminopyralid internal standard. With the use of the internal standard, matrix-matched standards were not used.

Method Changes

Two changes were made to the instrumental method from the original method provided. (1) The gradient was extended from 5.0 to 6.0 minutes and (2) the internal standard mass transition was changed from 269 → 195 to 269 → 111. These two changes were made as they presented a more optimized instrumental method for the analysis of aminopyralid and its internal standard. It's within reason to assume these changes could be due to the different manufacturers and models of HPLC-MS/MS instrumentation used here versus that used in the method validation.

Table 4 Instrument Conditions and Parameters

HPLC Conditions			
Chromatographic System:	Shimadzu Nexera XR		
Column:	Acquity UPLC HSS T3; 1.8 µm, 2.1 × 100 mm S/N: 02283930915105		
Temperature:	40 °C		
Flow rate (µL/min)	600		
Gradient:	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
	0.0	45	55
	0.5	45	55
	2.0	40	60
	2.5	5	95
	3.5	5	95
	4.0	45	55
	6.0	45	55
Mobile Phase A:	0.1% formic acid in water		
Mobile Phase B:	0.1% formic acid in acetonitrile		
Injection Volume:	20 µL		

MS/MS Conditions						
Detection System:	AB BioSystems/MDS Sciex API 6500+ LC/MS/MS					
Ionization:	Turbo Ion Spray					
Polarity:	Positive					
Curtain gas (CUR):	20.00					
Temperature (TEM):	600 °C					
Collision gas setting (CAD):	10.00					
GS1:	70.00					
GS2:	65.00					
IS:	5500					
Entrance potential (EP):	10.00					
Scan type:	MRM					
MRM Conditions	Transition (m/z)	Dwell (msec)	DP	CE	CXP	Retention Time (min)
Aminopyralid	262.9 → 133.9	400	80	57	13	1.9
	264.9 → 135.9					
Aminopyralid IS	268.9 → 111.0	400	16	31	14	1.9

Appendix D. ANALYTICAL METHOD

STUDY TITLE

Method Validation Study for the Determination of Residues of Aminopyralid in Compost by Liquid Chromatography with Tandem Mass Spectrometry

DATA REQUIREMENTS

OCSPP 850.6100
EU Council Regulation (EC) 1107/2009
SANCO/3029/99 rev. 4
SANCO/825/00 rev. 8.1
Dir98-02

Method Validation Study for the Determination of Residues of Aminopyralid in Compost by Liquid Chromatography with Tandem Mass Spectrometry

INTRODUCTION

Scope

This method is applicable for the quantitative determination of residues of aminopyralid in compost. The method was validated over the concentration range of 0.500 – 40.0 ng/g with a validated limit of quantitation of 0.500 ng/g. Common name, chemical name, and molecular formula for the analyte are given in Table 1.

This study was conducted to fulfill data requirements outlined in the EPA Residue Chemistry Test Guidelines, OCSPP 850.6100 (*1*). The validation also complies with the requirements of EU Council Regulation (EC) 1107/2009 with particular regard to Section 4 of SANCO/3029/99 rev.4 and Section 5 of SANCO/825/00 rev.8.1 as well as PMRA Residue Chemistry Guidelines as Regulatory Directive Dir98-02 (*2-4*). The validation was conducted following Dow AgroSciences SOP ECL-24.

Method Principle

Residues of aminopyralid were extracted from compost by shaking with 20 mL of 0.1N aqueous sodium hydroxide. The samples were then centrifuged. An aliquot of the sample was taken, acidified, and hydrolyzed in an oven at 90°C for 90 minutes. Upon cooling, samples were centrifuged again, and the supernatant purified via an Oasis MAX SPE procedure. After elution and drying down, internal standard (IS) was added, and samples were derivatized. The final, extracts were analyzed for derivatized aminopyralid using two structurally characteristic MS/MS transitions by liquid chromatography with tandem mass spectrometry.

Test Substance/Reference Compounds/Analytical Standards

Test Substance	TSN	Percent Purity	Recertification Date	Reference
Aminopyralid	TSN306691	99.0%	24-Jun-2022	FAPC18-000449

In addition, the compound below was used as an internal standard.

Test Substance	TSN	Percent Purity	Recertification Date	Reference
M+4 Aminopyralid	TSN314617	100%	08-Sep-2022	FAPC17-000489

The Certificates of Analysis for the test substance and internal standard can be found in Figures 1-2. The above standards may be obtained free of charge from Dow AgroSciences LLC, 9330 Zionsville Road, Building 304, Indianapolis, IN 46268-1054.

EXPERIMENTAL

Sample Origin, Numbering, Preparation, Storage, and Characterization

Untreated control samples were obtained from the Dow AgroSciences LLC Sample Management Group. All samples were tracked in the Dow AgroSciences LLC Regulatory Labs Information Management System (RLIMS) database. Unique sample numbers were assigned to the samples to track them during receipt, preparation, storage, and analysis. Complete source documentation was included in the study file.

The samples were prepared by freezing with dry ice and then grinding using a Robot Coupe bowl grinder with a 3 L, 30 L, or 45 L capacity bowl.

During the course of the study, the samples were stored in temperature-monitored freezers at approximately -20 °C, except when removed for analysis.

Determination of Isotopic Crossover

In this assay, the analyte and internal standard are quantified 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 separately at any analytically relevant concentration and if observed, should be addressed for accurate determination of analyte concentrations.

To determine the contribution of the unlabeled aminopyralid to the aminopyralid internal standard, a sample spiked with aminopyralid at the highest calibration standard without internal

standard in neat solution was prepared. The peak area in the internal standard transition was less than 5.0% of the internal standard peak area of the highest calibration standard spiked with internal standard.

In a similar manner, to determine the contribution of the labeled aminopyralid internal standard to the unlabeled aminopyralid, a sample was spiked at the working concentration of internal standard in neat solution that was not spiked with aminopyralid. The peak area in the analyte transition from the internal standard solution was less than the analyte peak area in the lowest calibration standard.

During method development, the concentration range of the calibration curve and the concentration of the labeled internal standard were chosen to minimize the effect of the crossover contribution between the analyte and internal standard. As a result, no significant mass spectral isotopic crossover was observed.

Calculation of Standard Calibration Curve

Calculation of a standard curve begins with the injection of a series of calibration standards described in Appendix I and acquisition of peak areas for the following analyte and internal standard.

Aminopyralid	*m/z* Q1/Q3 263/134 (quantitative) *m/z* Q1/Q3 265/136 (confirmatory)
Aminopyralid IS	*m/z* Q1/Q3 269/195

The linearity of detector response was evaluated using neat standard solutions. In order to generate a standard curve, the analyte concentration was plotted on the abscissa (x-axis) and the respective quantitation ratio on the ordinate (y-axis) in Analyst 1.6.3. Using regression analysis, the equation for the curve was determined with respect to the abscissa.

Confirmation of Residue Identity

The method is specific for the determination of aminopyralid by virtue of the chromatographic separation and selective detection system used. To demonstrate further confirmation, an additional MS/MS ion transition was monitored.

Statistical Treatment of Data

Statistical treatment of data included, but was not limited to, the calculation of regression equations, correlation coefficients (r) for describing the linearity of calibration curves, and means, standard deviations, and relative standard deviations of the results for the fortified recovery samples.

the primary identified component as parent aminopyralid, with its conjugates having been released as free aminopyralid upon exposure to hydrolytic conditions. The extraction and hydrolytic conditions implemented in this analytical method mirror those used to support the ^{14}C-metabolism studies. This method incorporates the use of a sodium hydroxide extraction solvent that serves to both extract and hydrolyze base-labile conjugates to free aminopyralid for quantitation. After which, the samples are acidified with hydrochloric acid and incubated at 90°C to hydrolyze acid-labile conjugates to free aminopyralid. Combined, these conditions allow for quantitative analysis of aminopyralid (free and conjugated residues), as free aminopyralid.

Calculated Limits of Detection and Quantitation

The limits of detection (LOD) and quantitation (LOQ) were proposed at the initiation of the study at 0.150 ng/g and 0.500 ng/g, respectively. Following established practices (*8*), the LOD and LOQ for the determination of residues of aminopyralid in compost were calculated using the standard deviation derived from the 0.500 ng/g recovery values. The results are summarized in Table 13. The validation results support the limits of detection and quantitation proposed for the study.

For actual residue samples, numerical results should be reported for residues that are equal to or above the LOD but less than the validated LOQ with indication that the results are being reported at a lower confidence level. For residues less than the LOD, results should be reported as not detected (ND).

Carryover

During the course of the validation, carryover was assessed for all analytes by injecting a solvent blank after the highest concentration standard, near the end of each analytical set. In addition, for four of the five analytical sets which comprise this validation, a solvent blank was similarly injected immediately after the second-highest concentration standard, closer to the beginning of each analytical set. For aminopyralid, no significant analyte response was detected in any solvent blank for each of these sets. !
except that a solvent blank was not injected immediately after the second-highest concentration standard, closer to the beginning of each analytical set. Instead, close to the beginning of that run, a solvent standard containing only IS was injected immediately after a solvent standard containing only the highest standard concentration of aminopyralid. The solvent standard containing only IS did not yield significant aminopyralid response from carryover, which is consistent with the other carryover data collected as part of this study.

Stability of Working Solutions

Analytical standards and fortification solutions were stored under refrigerated conditions when not in use. Stock standard and spiking standard stabilities were assessed in a separate study, and solutions of aminopyralid were found to be stable in acetonitrile for at least 198 days (*9*).

Stability of Sample Extracts

The stability of final samples prepared for analysis from representative matrices were evaluated over an extended period. Analytical sets were initially analyzed for pasture compost and manure compost on 21-Nov-2019 (Day 0), with average recovery values between 70-120% for the quantitative and confirmatory transitions. The final samples in the vials were then stored in the refrigerated autosampler at approximately 10°C. After 4 days, the LOQ samples were re-injected on 25-Nov-2019 using the same instrumental conditions described in the Instrumental Conditions section with freshly prepared calibration standards.

Sample extracts from pasture compost and manure compost were tested after 4 days of storage at refrigerated temperature and were determined to be stable.

Matrix Effects

Matrix effects were evaluated by comparing the response of the analyte fortified in a control extract after processing (for each matrix type) to the response of the analyte fortified in neat solvent. The calculation for the matrix effect based on peak area is as follows:

$$\text{Matrix Effect} = \left[\frac{\text{Average Peak Area}_{\text{Control Sample Spiked After Processing}}}{\text{Average Peak Area}_{\text{Spiked Neat Solvent}}} - 1\right] \times 100\%$$

To demonstrate that the use of the internal standard diminishes the extent of matrix effect, the calculation for the matrix effect based on area ratio is as follows:

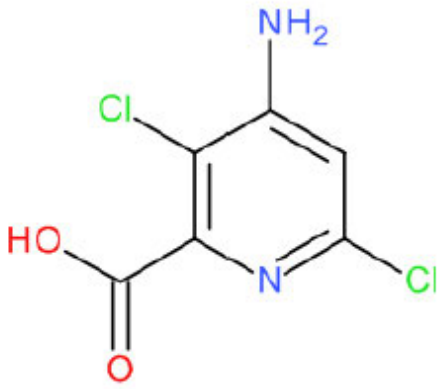
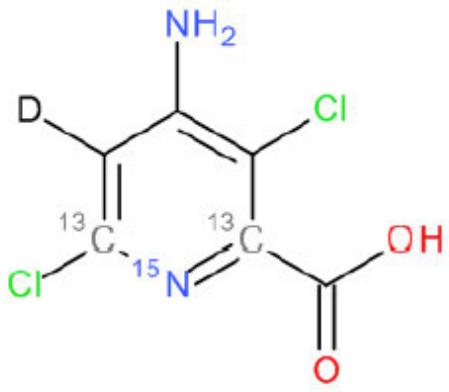
$$\text{Matrix Effect} = \left[\frac{\text{Area Ratio}_{\text{Control Sample Spiked After Processing}}}{\text{Area Ratio}_{\text{Spiked Neat Solvent}}} - 1\right] \times 100\%$$

A negative value for the matrix effect indicates matrix suppression, and a positive value for matrix effect indicates matrix enhancement.

The experimental details regarding determination of the matrix effects were recorded in the raw data file. Three aliquots of the control samples from each matrix were processed according to the method and followed the procedure. After the evaporation step following the Oasis MAX SPE procedure, each sample was fortified with an appropriate amount of spiking solution and IS, and derivatized. After derivatization, samples were diluted slightly with the addition of aqueous 0.1% formic acid. For comparison, three replicates of derivatized aminopyralid containing IS were prepared without matrix at the same concentration.

Matrix effects for the quantitative and confirmatory transitions were calculated using only analyte peak area as well as with the internal standard (area ratio).

Table 1. Identities and Structures of Aminopyralid and Aminopyralid Internal Standard

Identifying Information	Structure
Common Name of Compound: Aminopyralid Molecular Formula: $C_6H_4Cl_2N_2O_2$ Formula Weight: 207.01 CAS Number: 150114-71-9	
Common Name of Compound: M+4 Aminopyralid Molecular Formula: $C_4{}^{13}C_2H_3{}^{2}HCl_2N^{15}NO_2$ Formula Weight: 210.99 CAS Number: NA	

For example (Set 191576 S06), Sample 191576-001-0001A20 + 0.500 ng/g resulted in a peak area ratio of 0.0180 for Aminopyralid (263/134). Calculation is as follows:

$$y = 0.0368x + 0.000481 \ (r = 1.0000)$$

$$0.0180 = 0.0368x + 0.000481$$

$$0.0180 - 0.000481 = 0.0175 = 0.0368x$$

$$x = \frac{0.0175}{0.0368}$$

$$x = 0.476 \text{ ng/g} = \text{Aminopyralid (ng/g)}$$

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

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

$$\text{Recovery} = \frac{0.476 \text{ ng/g}}{0.500 \text{ ng/g}} \times 100\% = 95\%$$

Figure 5. Example Calculation for the Determination of Aminopyralid in Compost

APPENDIX I ANALYTICAL METHOD

ENFORCEMENT METHOD FOR DETERMINATION OF RESIDUES OF AMINOPYRALID IN COMPOST USING LIQUID CHROMATOGRAPHY WITH TANDEM MASS SPECTROMETRY

Scope

This method is applicable for the quantitative determination of aminopyralid in compost. This method is applicable over a concentration range 0.500 – 40.0 ng/g.

Principle

Residues of aminopyralid are extracted from the sample matrices by shaking with 0.1N NaOH. An aliquot of the extract is transferred. The sample is treated with an aliquot of concentrated (12.1 N) HCl and heated for 90 min at 90°C to hydrolyze any bound aminopyralid in the starting material. Samples are centrifuged to enable removal of some denser materials, and then undergo solid phase extraction. After internal standard (IS) is then added, samples are derivatized, nearly dried, and then reconstituted for analysis. Solvent calibration standards spiked with IS are also derivatized. Analysis occurs via liquid chromatography with positive ion electrospray ionization tandem mass spectrometry (LC-MS/MS).

Safety Precautions

Each analyst must be acquainted with the potential hazards of the equipment, reagents, products, solvents, and procedures used in this method before commencing laboratory work. Sources of information include operation manuals, material safety data sheets, literature, and other related data. Safety information should be obtained from the supplier. Disposal of waste materials, reagents, reactants, and solvents must be in compliance applicable governmental requirements.

Acetonitrile, isopropanol, ethyl acetate, 1-butanol, butyl chloroformate, pyridine, and methanol are flammable and should be used in well-ventilated areas away from ignition. Formic acid, trifluoroacetic acid, hydrochloric acid and sodium hydroxide are corrosive and can cause severe burns. It is imperative that proper eye and personal protection equipment be worn when handling these reagents.

Laboratory Equipment

Balance, analytical, Model AE240, Mettler-Toledo, Inc.

Balance, pan, Model PE1600, Mettler-Toledo, Inc.

2mL Clear Glass Flat Bottom Tubes, Catalog # 96VL20, Analytical Sales & Services

Centrifuge, Model Centra-GP8, Thermo International Equipment Company.

Pipette, positive-displacement, 10-25 µL capacity, Model M25, Gilson Inc.

Pipette, positive-displacement, 10-100 µL capacity, Model M100, Gilson Inc.

Pipette, positive-displacement, 50-250 µL capacity, Model M250, Gilson Inc.

Pipette, positive-displacement, 100-1000 µL capacity, Model M1000, Gilson Inc.

Repeater, positive-displacement, 1-50 mL capacity, catalog number 4982000322, Eppendorf.

Repeater Stream Pipette, 1-50 mL capacity, catalog number 4987000118, Eppendorf.

Shaker, variable speed reciprocating with box carrier, Model E6010.00, Eberbach Corporation.

Vortex mixer, Genie 2, catalog number 12-812, Fisher Scientific.

Chromatographic System

Column, analytical, Aquity UPLC HSS T3 1.8µm (2.1x100mm), catalog number 186003539, Waters.

Liquid chromatography, 1290 Infinity, Agilent.

Mass spectrometer, Model QTRAP 5500, AB SCIEX.

Mass spectrometer data system, Analyst v.1.6.3, AB SCIEX.

Pre-column filter, KrudKatcher ULTRA HPLC in-line filter, 0.5 µm depth filter x 0.004 in ID, catalog number AF0-8497, Phenomenex.

Glassware and Materials

Centrifuge tubes, 50 mL, HDPE, polypropylene with screw cap, catalog number 06-443-20, Fisher Scientific.

Culture Tube, Disposable, 16 x 100 mm, catalog number 14-961-29, Fisher Scientific.

Culture Tube, Disposable, screw top, 16 x 100mm, cat # 73770-16100, Kimble Chase.

Combitips Advanced, 0.5 mL, catalog number 0030089421, Eppendorf.

Combitips Advanced 1.0 mL, catalog number 0030089430, Eppendorf.

Combitips Advanced 5 mL, catalog number 0030089456, Eppendorf.

Combitips Advanced 10 mL, catalog number 0030089464, Eppendorf.

Combitips Advanced 25 mL, catalog number 0030089472, Eppendorf.

Combitips Advanced 50 mL, catalog number 0030089480, Eppendorf.

Filters, syringe, Acrodisc 13 mm w/ 0.2 µm PTFE, Pall Corporation.

Graduated cylinder, borosilicate glass, 100 mL, catalog number S63458, Fisher Scientific.

Graduated cylinder, borosilicate glass, 500 mL, catalog number S63460, Fisher Scientific.

Graduated cylinder, borosilicate glass, 1000 mL, catalog number S63461, Fisher Scientific.

Pipette, disposable, transfer, glass, 5 ¾ inch, catalog number 13-678-6A, Fisher Scientific.

Pipette, disposable, transfer, polyethylene, catalog number 13-711-7M, Fisher Scientific.

Pipette tip, positive-displacement, 25 µL capacity, catalog number CP25, Gilson Inc.

Pipette tip, positive-displacement, 100 µL capacity, catalog number CP100, Gilson Inc.

Pipette tip, positive-displacement, 250 µL capacity, catalog number CP250, Gilson Inc.

Pipette tip, positive-displacement, 1000 µL capacity, catalog number CP1000, Gilson Inc.

SPE column, Oasis MAX 6cc (150 mg) LP Extraction Cartridges, Part Number 186000370, Waters.

Syringe, 3 mL BD Luer Lok tip, Becton Dickinson.

Vial, autosampler, 2 mL, catalog number C4000-1W, Thermo Scientific.

Vial inserts, 400 µL, Target MicroSerts, National C4011-631, Thermo Scientific.

Vial cap, for autosampler vial, catalog number C5000-57G, Thermo Scientific.

Volumetric flask, 10 mL, catalog number K623010-0010, Fisher Scientific.

Volumetric flask, 20 mL, catalog number K623010-0020, Fisher Scientific.

Reagents

Acetonitrile, HPLC grade, catalog number A998-4, Fisher Scientific.

1-Butanol, HPLC grade, catalog number A383-1, Fisher Scientific.

Butyl Chloroformate, 98%, catalog number 180170010, ACROS Chemical.

Ethyl Acetate, ACS grade, catalog number 319902-4L, Sigma Aldrich.

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

Glycerol, catalog number G33-500, Fisher Scientific.

Hydrochloric Acid, concentrated (12.1 N), catalog number A144S-500, Fisher Scientific.

Isopropanol, HPLC grade, catalog number A464-4, Fisher Scientific.

Methanol, HPLC grade, catalog number A452-4, Fisher Scientific.

Pyridine, HPLC grade, catalog number 396800010, ACROS Organics.

Sodium Hydroxide 2N, certified concentration, catalog number SS264-1, Fisher Scientific.

Water, Optima LC/MS grade, catalog number W6-4, Fisher Scientific.

Prepared Solutions

Preparation volumes may be adjusted based on need. Care must be taken to ensure that the ratios and concentrations are maintained. Allow solutions to reach room temperature before use. Store at room temperature unless otherwise directed.

0.1% Formic Acid in Water (Mobile Phase A)
Measure 1000 mL of HPLC grade water by a 1-L graduated mixing cylinder. Add 1.0 mL of formic acid and mix. Mix well.

0.1% Formic Acid in Acetonitrile (Mobile Phase B)
Add 1000 mL of acetonitrile by graduated cylinder to a 1-L bottle. Add 1.0 mL of formic acid by pipette. Mix well.

0.1N Sodium Hydroxide (NaOH) - Extraction Solution
Measure 380 mL of HPLC grade water with a 500 mL graduated cylinder and transfer to a 500 mL bottle. Add 20.0 mL of 2 N sodium hydroxide to the 500 mL bottle. Mix thoroughly.

30/30/30/10, Methanol/Isopropanol/Acetonitrile/Water (v/v/v/v) (Needle Wash)
Measure 1200-mL of methanol, 1200-mL of isopropanol, 1200-mL of acetonitrile, and 400-mL of water, using a graduated cylinder, and transfer into a 4-L bottle. Mix.

Methanol/Water/Acetic Acid (50/49/1, v/v/v)
Measure 500 mL of methanol with a graduated cylinder and transfer to a bottle. Measure 490 mL water with a graduated cylinder and transfer to the same bottle. Pipette 10 mL of glacial acetic acid into the bottle. Mix thoroughly.

Ethyl Acetate/Trifluoroacetic Acid (98/2, v/v)

Measure 980 mL of ethyl acetate with a graduated cylinder and transfer to a bottle. Pipette 20 mL of trifluoroacetic acid into the bottle. Mix thoroughly.

1-Butanol/Glycerol (90/10, v/w)

Weigh 1 g of glycerol into a bottle. Measure 9.0 mL of 1-butanol with a graduated cylinder and transfer to the bottle. Mix thoroughly.

22:2:1 Acetonitrile: Pyridine: 1-Butanol (Derivitization Coupling Reagent)
Add 22 mL of acetonitrile, 2 mL of pyridine, and 1 mL of 1-butanol by pipette to a small glass jar. Mix well. Prepare fresh every 2 weeks.

90/10 Acetonitrile: Butyl Chloroformate (Derivitization Reagent)
Add 90 mL of HPLC grade acetonitrile and 10 mL of butyl chloroformate by pipette to a small glass jar. Mix well.

Preparation of Stock Solutions

Accurately weigh approximately 0.002 g of the aminopyralid analytical standard and dilute to volume with an appropriate amount of acetonitrile to obtain a 100 µg/mL aminopyralid stock solution.

Quantitatively combine 1.00 mL of the 100 µg/mL stock solution and 19.0 mL acetonitrile to obtain a 5000 ng/mL aminopyralid stock solution.

Quantitatively combine 2.00 mL of the 5000 ng/mL stock solution and 18.0 mL acetonitrile to obtain a 500 ng/mL aminopyralid stock solution.

Quantitatively combine 2.00 mL of the 500 ng/mL stock solution and 18.0 mL acetonitrile to obtain a 50.0 ng/mL aminopyralid stock solution.

Quantitatively combine 5.00 mL of the 50.0 ng/mL stock solution and 5.00 mL acetonitrile to obtain a 25.0 ng/mL aminopyralid stock solution.

Quantitatively combine 1.00 mL of the 50.0 ng/mL stock solution and 9.00 mL acetonitrile to obtain a 5.00 ng/mL aminopyralid stock solution.

Preparation of Intermediate Calibration STD Spiking Solutions

Prepare the following Intermediate Calibration STD Spiking Solutions in acetonitrile:

Concentration of Stock Solution (ng/mL)	Aliquot of Stock Solution (µL)	Final Solution Volume (mL)	Concentration of Intermediate Calibration STD Solution (ng/mL)	Intermediate Calibration STD Spiking Solution	*Equivalent* Neat Cal STD Concentration (ng/g)*
25.0	315	20.0	0.39375	A	0.150
25.0	630	20.0	0.7875	B	0.300
50.0	525	20.0	1.3125	C	0.500
50.0	1050	20.0	2.625	D	1.00
50.0	2100	20.0	5.25	E	2.00
500	525	20.0	13.125	F	5.00
500	1050	20.0	26.25	G	10.0
500	2100	20.0	52.5	H	20.0
5000	315	20.0	78.75	I	30.0
5000	525	20.0	131.25	J	50.0

*Note: Equivalent Sample Conc (ng/g) is based on 1 g of sample diluted in 20 mL, aliquot factor of 7.00 mL (7/20 of extracted sample). Overall method factor is 0.2625 {(7/20) * (3/4)}, as only 6 mL of 8 mL total hydrolysate volume is taken through SPE clean-up. Only 100 µL of STDs A – J are used to prepare neat calibration standards.

Preparation of Internal Standard

Accurately weigh approximately 0.002 g of $^{13}C_2{}^{2}H^{15}N$-aminopyralid internal standard and dilute to volume with an appropriate amount of methanol to obtain a 100 µg/mL aminopyralid internal standard solution.

Quantitatively combine 50.0 µL of the 100 µg/mL aminopyralid internal standard solution and 9.95 mL acetonitrile to obtain a 500 ng/mL aminopyralid internal standard solution.

Quantitatively combine 2.00 mL of the 500 ng/mL aminopyralid internal standard solution and 8.00 mL acetonitrile to obtain a 100 ng/mL aminopyralid internal standard solution.

Note: Weights and volumes may be adjusted for solution and standard preparation as long as the final concentrations are equivalent.

Analysis Procedure

For procedural recovery samples:

1. For reagent blank, add 20 mL of 0.1N NaOH extraction solution into an empty 50-mL centrifuge tube.
2. For control samples, transfer 1.00 ± 0.05 g of compost material into an empty 50-mL centrifuge tube.
3. For fortified recovery samples, transfer 1.00 ± 0.05 g of compost material into an empty 50-mL centrifuge tube. Add the appropriate volume of the spiking solution to obtain fortified samples.

To fortify 1.0 g of compost with Aminopyralid			
Sample	Spiking Volumes (µL)	Stock Solution Conc. (ng/mL)	Fortification Level (ng/g)
LOD	30.0	5.00	0.150
LOQ	100	5.00	0.500
10x LOQ	100	50.0	5.00
80x LOQ	80.0	500	40.0

For field samples:

4. Measure by weight, 1.00 ± 0.05 g of each compost sample into an empty 50-mL centrifuge tube.

For ALL samples:

5. Add 20.0 mL of extraction solution, 0.1N NaOH, to each 50-mL centrifuge tube and seal tightly with a cap. Vortex to mix.
6. Shake the sample for 60 min on a flatbed shaker set at approximately 280 excursions/minute.
7. Centrifuge the sample for 5 min at 3000 rpm. (Note: acceptable stopping point if sample is decanted into separate vials and kept refrigerated).
8. Pipette 7.00 mL of each extract supernatant into labeled 16mm screw cap glass tubes.
9. Add 660 µL concentrated (12.1N) HCl to each tube, cap and mix well.
10. Place the tubes in an oven or water bath at 90°C for 90 minutes.
11. Remove tubes from heat and allow to cool without removing screw caps.
12. Add 340 µL water to each tube, and mix well.
13. Centrifuge tubes at 3000 RPM for 10 minutes.
14. Use a disposable pipette to carefully transfer AT LEAST 6.5 mL supernatant (without disturbing any precipitate) into labelled scintillation vials (no cap needed).

15. Purify the samples with Oasis MAX SPE cartridges (6 mL, 150 mg) using the following procedure.
    a. Condition all cartridges with 4 mL of MeOH. Discard the eluate.
    b. Equilibrate all cartridges with 4 mL of water. Discard the eluate.
    c. Accurately load 6.00 mL of each sample onto the SPE cartridge. Aim for an elution rate of about 1-2 seconds between drops. Discard the eluate.
    d. Rinse each cartridge with two 3 mL aliquots of water. Aim for an elution rate of about 1-2 seconds between drops. Discard the eluate.
    e. Wash the cartridge with two 4 mL aliquots of methanol/water/acetic acid (50/49/1, v/v/v). Aim for an elution rate of about 1-2 seconds between drops. Dry the SPE cartridge after the final aliquot for at least 30 seconds. Discard the eluate.
    f. Elute with two 3.50 mL aliquots of ethyl acetate/trifluoroacetic acid (98/2, v/v) into labelled 16 x 100 mm culture tubes containing 20 µL of 1-butanol/glycerol (90/10, v/w) solution. Aim for an elution rate of about 1-2 seconds between drops.
16. Evaporate to dryness on a Turbovap set at 40°C using a gentle stream of nitrogen. Adjust nitrogen flow as evaporation occurs ranging from 0.5 L/min to 1.2 L/min; the samples should be dry in approximately 60 min. For preparation of neat calibration standards, continue to step 17. For all other samples, skip to step 18.
17. For neat calibration standards, aliquot 100 µL of each Intermediate Calibration STD Spiking Solution (A – J) into separate, labeled, clean test tubes.
18. Add 50.0 µL of 100 ng/mL IS in ACN to each sample. Vortex-mix.
19. Evaporate all samples to dryness on a Turbovap set at 40°C with a gentle stream of nitrogen (about 1.0 L/min). **[Note: It is vital that <u>any</u> and <u>all</u> alcohol and water remaining from the previous step(s) be completely dried off before adding the derivatizing agents in the next two steps.]**
20. Add 200 µL of the acetonitrile/pyridine/1-butanol (22/2/1, v/v/v) solution, to all vials. Vortex to mix. Sonicate briefly to re-dissolve dried extract.
21. Derivatize the samples and the calibration standards by pipetting 100 µL of the acetonitrile/butyl chloroformate (90/10, v/v) solution into the tubes.
22. Immediately vortex mix the samples and the standards for a few seconds. Then allow the mixtures to react at room temperature for about 15 minutes.
23. Add 250 µL 0.1% formic acid in water to each sample and mix well. Sonicate about 30 seconds.

24. Using a syringe fitted with a filter (13 mm, 0.2 µm PTFE), transfer samples to low volume autosampler vials, or autosampler vials with low volume glass inserts. If using inserts, this is most easily accomplished by filtering directly into the wide-mouthed autosampler vial, and then using a pipette to transfer filtered extract into the low volume insert.
25. Analyze the derivatized calibration standards and samples by HPLC with positive-ion electrospray tandem mass spectrometry.

Chromatography

Determine the suitability of the chromatography system using the following performance 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 examine the chromatograms with respect to peak response, baseline noise, and background interference.

Supplemental Notes

Equipment, glassware, materials, reagents, and chemicals considered to be equivalent to those specified may be substituted with the understanding that their performance must be confirmed by appropriate tests. Common laboratory glassware and supplies are assumed to be readily available. Unless specified otherwise, class A volumetric glassware is used to prepare analytical standards, fortification solutions, and calibration standards.

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

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

Instrumental Conditions

Typical LC-MS/MS Operating Conditions for Determining Aminopyralid in Compost

Instrumentation: Agilent 1290 Infinity LC System
AB SCIEX QTRAP 5500 LC/MS/MS System
AB SCIEX Analyst 1.6.3 data system

Column: Waters HSS T3, 2.1 x 100 mm, 1.8 µm
Column Temperature: 40 °C
Sample Temperature 10 °C
Injection Volume: 20 µL
Autosampler Wash 30 seconds of 30/30/30/10 Methanol/ Isopropanol/ Acetonitrile/ Water (v/v/v/v) at the flush port
Run Time: approximately 5 minutes
Mobile Phase: A – water containing 0.1 % formic acid
B – acetonitrile containing 0.1 % formic acid

Gradient:

Time, min	Flow Rate (µL/min)	Solvent A, %	Solvent B, %
0.0	600	45	55
0.5	600	45	55
2.0	600	40	60
2.5	600	5	95
3.5	600	5	95
4.0	600	45	55
5.0	600	45	55

Flow Diverter
1) 0.0 → 1.4 min – flow to waste
2) 1.4 → 2.5 min – flow to MS
3) 2.5 → end of run - flow to waste

Typical Mass Spectrometry Operating Conditions for Determining Aminopyralid in Compost

Ionization Mode:	Electrospray
Polarity:	Positive
Scan Type:	MRM
Resolution:	Q1 – unit, Q3 – unit
Collision Gas (CAD):	10
Curtain Gas (CUR):	20
Ion Source Gas 1 (GS1):	50 psi
Ion Source Gas 2 (GS2):	50 psi
Temperature (TEM):	600 °C
Entrance Potential (V):	10
IonSpray Voltage (IS):	5500 volts
Acquisition Duration (min):	2.0
Dwell Time (ms):	50

MS Conditions:

	Precursor Ion Q1 (*m/z*)	Product Ion Q3 (*m/z*)	Declustering Potential (V)	Collision Energy (V)	Cell Exit Potential (V)
Aminopyralid (263/134)	262.9	133.9	80	57	13
Aminopyralid (265/136)	264.9	135.9	80	57	13
Aminopyralid IS (269/195)	269.0	195.0	80	27	13

Aminopyralid transition 263/134 is used for quantitation. Aminopyralid transition 265/136 is used for confirmation. Use of 263/161 with CE = 39 volts as a confirmation transition yielded too much interference with some matrices at the very low end of the range.

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