STUDY TITLE

Independent Laboratory Validation of "Method Validation Study for the Determination of Residues of Picloram in Compost by Liquid Chromatography with Tandem Mass Spectrometry"

DATA REQUIREMENTS

OPPTS 860.1340
OCSPP 850.6100

STUDY COMPLETED ON

07-Dec-2018

Independent Laboratory Validation of "Method Validation Study for the Determination of Residues of Picloram in Compost by Liquid Chromatography with Tandem Mass Spectrometry"

INTRODUCTION

Scope

This method is applicable for the quantitative determination of residues of picloram in compost. The method was independently validated over the concentration range of 1.0-10 ng/g with a validated limit of quantitation of 1.0 ng/g. Common names, chemical names, and molecular formulas for the analyte are given in Table 1.

This study was conducted to fulfill data requirements outlined in the EPA Residue Chemistry Test Guidelines, OPPTS 860.1340 (*1*) and OCSPP 850.6100 (*2*). The validation was conducted following EAG SOPs, with exceptions noted in the protocol amendment.

Method Principle

Residues of picloram are extracted from samples by vigorously shaking for at least 60 minutes with methanol/10N sodium hydroxide (100/1, v/v). The extract is allowed to sit overnight (minimum of 12 hours) followed by centrifugation. An aliquot of the supernatant is evaporated to dryness, reconstituted in 0.2N HCl, and vortexed. The sample extracts are purified via HLB Solid Phase Extraction (SPE) and AFFINIMIP SPE. The sample extracts are evaporated to dryness, derivatized using ACN:ethyl chloroformate (9:1, v:v), and analyzed for picloram by tandem liquid chromatography coupled with mass spectrometry (LC-MS/MS).

Test Substances/Reference Compounds/Analytical Standards

Test Substance	TSN	Percent Purity	Recertification Date	Reference
Picloram	TSN029006-0001	99.7%	04-Jun-2026	FAPC14-000119
Picloram IS (X11050262)	TSN314292	100%	16-Jun-2022	FAPC17-000330

EXPERIMENTAL

Sample Origin, Numbering, Preparation and Storage

The untreated control sample was received from Dow AgroSciences LLC on 06-Sep-2018. The sample was entered in the EAG Labs Information Management System (LIMS) database. A unique sample number was assigned to the sample to track its receipt and storage. Complete source documentation was included in the study file.

The compost sample was received from the Sponsor already homogenized, so no further sample preparation was required.

During the course of the study, the sample was stored in temperature-monitored freezers at approximately -20 °C, except when removed for analysis. Complete documentation for the compost source may be found in the raw study file.

Instrumentation

An Applied Biosystems/Sciex API 5500 Q-Trap Mass Spectrometer LC-MS/MS system, with Waters Acquity HPLC System, in conjunction with Applied Biosystems/MDS Sciex Analyst Software for data collection and system control (version 1.6.2) was used for analysis of the data.

Column: Acquity UPLC HSS T3, 2.1 x 100 mm, 1.8 µm

In-line Filter: Phenomenex KrudKatcher Ultra, 0.5 µm depth filter x 0.004 in ID

Mobile Phase: Component A: 0.1% Formic Acid (aq)

Component B: 0.1% Formic Acid in Acetonitrile

Gradient:

Time (min.)	% A	% B	Flow Rate (mL/min)
0.00	95	5	0.300
2.00	60	40	0.300
9.00	54	46	0.300
14.6	5	95	0.300
15.6	5	95	0.300
16.6	95	5	0.600
20.7	95	5	0.600
21.7	95	5	0.300

Run Time: 21.7 minutes

Diverter Program:	0-6.0 minutes	Flow to Waste
	6.0-10.5 minutes	Flow to Source
	10.5-21.7 minutes	Flow to Waste

Injection Volume:	5 µL
Column Temperature:	15 °C

Typical Mass Spectrometer Conditions

Acquisition Time:	10 minutes 60 seconds
Polarity:	Positive
Scan Type:	MRM
Resolution:	Q1, Q3 Unit
Curtain Gas (CUR):	40.00
Collision Gas (CAD):	"High"
Temperature (TEM):	400 °C
Ion Source Gas 1 (GS1):	50.00
Ion Source Gas 2 (GS2):	50.00
IonSpray Voltage (IS):	5500.00
Entrance Potential (EP):	10.00
Declustering Potential (DP):	110.00
Cell Exit Potential (CXP):	10.00

Ions Monitored:	Picloram	Collision Energy (CE):
Quantitation:	*m/z* 269/168	46
Confirmation (primary):	*m/z* 271/143	65
Internal Standard:	*m/z* 274/144	65
Dwell Time:	200 msec	

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 picloram at *m/z* 269 (quantitation) and *m/z* 271 (confirmation). Matrix-matched standards were used to calculate the sample results, while a solvent standard curve was used to calculate the results for the reagent blank.

The linearity of detector response was evaluated using matrix-matched standard solutions for samples, and solvent standard solutions for the reagent blank. In order to generate standard

curves, the analyte concentration was plotted on the abscissa (x-axis), and the respective peak area was plotted on the ordinate (y-axis) using Analyst 1.6.2 to perform 1/x regression analysis.

Confirmation of Residue Identity

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

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, means, standard deviations, and relative standard deviations of the results for the fortified recovery samples plus LOD and LOQ values.

RESULTS AND DISCUSSION

Assay Time

A typical analytical run consisted of a minimum of seven calibration standards (at different concentration levels) with a range from 30% of the LOQ to 30% above the highest sample fortification level, a reagent blank, two controls (non-fortified samples), and eleven fortified controls. This typical sample set can be taken through the analytical procedure in approximately 13.5 hours (approximately one and one-half business days) by implementing overnight sample hydrolysis followed by workup of results the following day.

Critical Steps

There were no critical steps encountered during this independent method validation.

Specificity of Method and Confirmation of Residue Identity

The method is selective for the determination of picloram by virtue of the chromatographic separation and MS detection. Significant peak response (>30% of the LOQ peak area) is not observed in reagent blank and extracts of untreated blank control samples at the expected retention time of the analyte. Unambiguous identification is ensured by the comparison of retention time of recovery samples with the retention time of the calibration standards as well as by monitoring two structurally characteristic fragment ions by mass spectrometry.

Validation data obtained using the confirmatory MS transition met the same acceptance criteria as the validation data generated using the quantitative MS transition, therefore demonstrating

that the analyte signal of the quantitative MS transition was correct and not affected by any other compound.

Ruggedness of Method

For compost, validation analyses were conducted over the course of two days, including an overnight hydrolysis. The analysis results were all acceptable, demonstrating the ruggedness of the method.

Full-Scan and Product-Ion Mass Spectra

Full scan mass spectra of picloram and its internal standard are illustrated in Figure 8.

Standard Curve Linearity

The linearity of detector response for samples was evaluated using matrix-matched standard solutions; the linearity of detector response for the reagent blank sample was evaluated using solvent standard solutions. All calibration curves were calculated by linear regression analysis with 1/x weighting. Calibration curves resulting from the injection of seven standards over the concentration range of 0.150-50.0 ng/mL

Calculated Limits of Detection and Quantitation

The limits of detection (LOD) and quantitation (LOQ) were proposed at the initiation of the study at 0.3 ng/g and 1.0 ng/g, respectively. Following established practices (3), the LOD and LOQ for the determination of residues of picloram in compost samples were calculated using the standard deviation derived from the 1.0 ng/g recovery values. The results are summarized in Table 10. The validation results support the limits of detection and quantitation proposed for the study.

Stability of Stock, Fortification, Intermediate Solutions, and Calibration Standards

As part of this independent laboratory validation study, the stability of the stock, fortification, and intermediate solutions, in addition to the calibration standards was evaluated over a period of 14-20 days. The results indicate that picloram stock and fortification solutions prepared in methanol are stable for at least 15 days when stored at 2-8 °C. Picloram intermediate and calibration standard solutions prepared in methanol are stable for at least 15-20 and 14 days, respectively, when stored at 2-8 °C (Table 13).

Stability of Sample Extracts

All sample extracts for this study were injected within approximately 72 hours of extraction.

Matrix Effects

Matrix effects were evaluated by comparing the response of the analyte fortified in a processed control extract to the response of the analyte fortified in neat solvent. The calculation for the matrix effect is as follows:

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

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. An aliquot of the control sample was processed following the method. After the evaporation step, each sample was derivatized using 200 µL of ACN:ethyl chloroformate (9:1, v:v).

Changes to the Method

A number of the solvents, reagents and equipment (i.e. vortex mixer, centrifuge, etc.) were not identical to those used in the analytical method provided; however, they were deemed equivalent and acceptable for use during the successful control suitability. There were, however, two changes made during this independent method validation that were felt to be more significant:

1. An N-evap nitrogen evaporator with heated water bath from Organomation Associates, Inc. was used rather than the TurboVap LV evaporator from Biotage for method step 11.11. This change was approved by the Sponsor Representative prior to Trial 1.
2. While this was not a change to the method, a suggestion would be to note that solvents/solutions are eluted with a vacuum, not gravity, for the HLB SPE procedure; specifically, method steps 11.14.1→11.14.4. A communication with the Sponsor was required to verify this information prior to Trial 1.
3. The Laboratory Equipment Section of the analytical method appears to be missing the centrifuge.
4. In Section 8, Preparation of Spiking Solutions, a 1-mg/mL stock was prepared rather than a 10.0-mg/mL to allow for preparation of a second 1-mg/mL stock at the end of the study for standard stability assessment. This change was not discussed with the Sponsor, but arose based on the limited amount of reference material supplied.
5. In the "note" that appears in method step 11.17.1, it appears that the referenced method step should be 11.10 rather than 11.9.

CONCLUSIONS

The analytical method for the determination of picloram in compost has been demonstrated to be satisfactory in terms of accuracy, precision, linearity, and specificity based on the analysis of Set ILV1. The method was validated over the concentration range of 1.0-10 ng/g with a limit of quantitation of 1.0 ng/g.

Table 1. Identities and Structures of Picloram and its Internal Standard

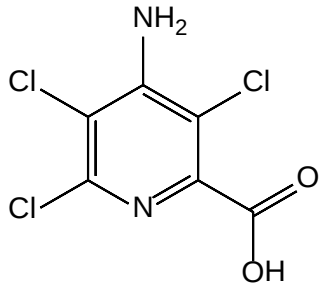
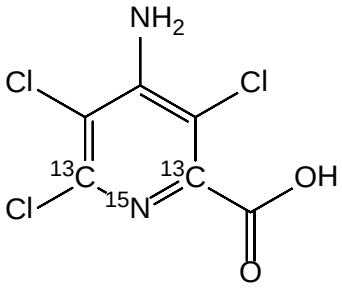
Common Name	Structural Formula and Chemical Name
Picloram Molecular Formula: $C_6H_3Cl_3N_2O_2$ CAS Number: 1918-02-1	 4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid
Picloram IS (X11050262) Molecular Formula: $C_4{}^{13}C_2H_3Cl_3N^{15}NO_2$ CAS Number: NA	 4-amino-3,5,6-trichloropyridine-2-carboxylic acid-1-^{15}N-2, 6-^{13}C

Table 10. Calculated Limits of Detection and Quantitation for Picloram

Analyte	Matrix	Average ng/g Found[a]	Standard Deviation	Calculated Limit of Detection	Calculated Limit of Quantitation	Number of Samples
Picloram Quantitation (*m/z* 269)	Compost	0.99670	0.04236291	0.158734	0.47620	5
Picloram Confirmation (*m/z* 271)	Compost	1.01530	0.06181926	0.231637	0.69491	5

LOD = $t_{0.99}$ (3.747) * Standard Deviation
LOQ = LOD * 3
[a] 1.0 ng/g fortification

Table 11. Matrix Effects on Picloram (*m/z* 269) for Quantitative Transition

Matrix Type	Picloram (*m/z* 269)	
	Peak Area	Matrix Effect (%)
Compost	32363	
Neat Solvent	35649	

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

Table 12. Matrix Effects on Picloram (*m/z* 271) for Confirmatory Transition

Matrix Type	Picloram (*m/z* 271)	
	Peak Area	Matrix Effect (%)
Compost	31930	
Neat Solvent	44046	

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

Linear 1/x
Equations

Calculations for instrumental analysis were conducted using Analyst 1.6.2 software to create a standard curve based on linear regression. Regression functions were used to calculate a best-fit line [from a set of standard concentrations (in ng/mL) versus analyte peak area/IS peak area (in peak response ratio)] and to determine concentrations of the analyte(s) found (in ng/mL) during sample analysis from the calculated best-fit line.

The following equation was used to determine the least squares fit:

$$y = mx + b$$

where:

y = peak response ratio of analyte to IS
m = slope
x = ng/mL found for peak of interest
b = y-intercept

The following equation was used to calculate the amount of analyte in ppm found in the sample:

$$ng/g = ng/mL\ Found \times \frac{Final\ Volume\ (mL) \times Extract\ Volume\ (mL)}{Sample\ Wt\ (g) \times Aliquot\ Volume\ (mL)} \times Dil.\ Factor$$

where:

ng/g = ng/g analyte(s) found in sample
ng/mL = ng/mL of analyte(s) found in sample calculated from standard curve
Sample Wt (g) = gram weight of sample extracted (1.00 g)
Extract Volume (mL) = volume of extraction solvent (20 mL)
Aliquot Volume (mL) = volume of aliquot taken (5 mL)
Final Volume (mL) = volume of final extract submitted to LC-MS/MS (0.500 mL)
Dil. Factor = dilution of sample extract required to produce an analyte response bracketed by standards

The following equation was used to calculate the percent recovery for fortified samples:

$$\%\ Rec = \frac{ng/g\ found\ in\ fortified\ sample}{ng/g\ added} \times 100$$

Example Calculations

Fortified Control Data
Set ID = ILV1
Analyte = Picloram (Quantitation; *m/z* 269)
Sample ID = 87370-005, 141184-007-0003A5 + 1.0 ng/g (LOQ)
Peak Response Ratio = 34216/127645 = 0.26805
Slope = 0.4030867
y-intercept = 0.06420437

Figure 7. Example Calculation for the Quantitative Determination of Picloram in Compost (Set ILV1, 87370-005)

Fortified Control Example

$$\text{ng/mL Found} = \frac{0.26805 - 0.06420437}{0.4030867}$$

$$= 0.50572 \text{ ng/mL, Reported as } 0.50572 \text{ ng/mL}$$

$$\text{ng/g} = 0.50572 \text{ ng/mL} \times \frac{0.500 \text{ mL} \times 20 \text{ mL}}{1.00 \text{ g} \times 5 \text{ mL}} \times 1$$

$$= 1.01144 \text{ ng/g, Reported as } 1.01 \text{ ng/g}$$

$$\%\text{ Rec} = \frac{1.01 \text{ ng/g}}{1.0 \text{ ng/g}} \times 100$$

$$= 101\%$$

Figure 7. Example Calculation for the Quantitative Determination of Picloram in Compost (Set ILV1, 87370-005) (continued)

APPENDIX I ANALYTICAL METHOD

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

1. Scope

This method is applicable for the quantitative determination of picloram in compost. The method concentration range for picloram will be 1.0-1,000,000 ng/g with a targeted limit of quantitation of 1.0 ng/g.

2. 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, ethanol, ethyl 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.

3. Laboratory Equipment

Balance, analytical, Model XPE205, Mettler-Toledo, Inc.

Balance, pan, Model ML3002E/03 and ML3002E, Mettler-Toledo, Inc.

Centrifuge, with rotor to accommodate 50-mL polypropylene centrifuge tubes, Sorvall Model Legend XTR, Thermo Scientific.

Evaporator, N-Evap, Model N116, Organomation Associates, Inc.

Evaporator, TurboVap LV, Zymark.

Pipet, positive-displacement, 3-25 µL capacity, Model M25, Gilson Inc.

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

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

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

Repeater Xstream Pipet, 1 µL-10 mL capacity, Eppendorf.

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

Sonicator, Model 8800, Branson.

Vacuum manifold, Visiprep SPE, Sigma Aldrich.

Vortex mixer, Maxi Mix I, Thermo Scientific.

4. Chromatographic System

Column, analytical, Acquity UPLC HSS T3 1.8 µm (2.1 x 100 mm), catalog number 186004056, Waters.

In-Line filter, KrudKatcher Ultra (0.5 µm depth filter x 0.004 in ID), catalog number AF0-8497, Phenomenex.

Liquid chromatograph, Acquity UPLC, Agilent.

Mass spectrometer, Model QTRAP 5500, AB SCIEX.

Mass spectrometer data system, Analyst v.1.6.2, AB SCIEX.

5. Glassware and Materials

Centrifuge tube, 50 mL, Falcon™, polypropylene with screw cap, part number 14-959-49A, Fisher Scientific.

Combitips Advanced 1.0 mL, catalog number 0030089430, Eppendorf.

Combitips Advanced 2.5 mL, catalog number 0030089448, Eppendorf.

Combitips Advanced 5 mL, catalog number 0030089456, Eppendorf.

Combitips Advanced 10 mL, catalog number 0030089464, Eppendorf.

Combitips Advanced 50 mL, catalog number 0030089480, Eppendorf.

Pipet, Class A, glass, 2.0 mL, 2.5 mL, 5.0 mL, and 7.0 mL, Fisher Scientific.

Pipet, Serological, glass, 1 mL, 5 mL, 10 mL, and 25 mL, Fisher Scientific.

Culture Tube, glass, 16 x 125 mm, part number 14-959-35A, Fisher Scientific.

Teflon-lined screw top to fit 16 x 125mm culture tube, part number 02-913-109, Qorpak.

Glass pasteur pipet, 5.75 in. length, catalog number 13-678-6A, Fisher Scientific.

Graduated cylinder, borosilicate glass, 25 mL, 50 mL, 100 mL, 500 mL, 1000 mL, and 2000 mL, Fisher Scientific.

Graduated mixing cylinder, glass, 250 mL, Fisher Scientific.

pH strips, pH 0-14 MColorpHast™, Millipore.

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

Pipet tip, positive-displacement, 3-25 µL capacity, Model M25, Gilson Inc.

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

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

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

SPE column, AFFINIMIP SPE Picolinic Herbicides, 6 mL, 100 mg sorbent, 50/box, part number, FS115-03b, AFFINISEP.

SPE column, Oasis HLB, 200 mg, 6 mL, part number WAT106202, Waters.

Vial, autosampler, 2 mL, 11 mm amber glass crimp/snap top, catalog number C4011-6W, Thermo Scientific.

Autosampler vial insert, 300 µL, catalog number 03-375-3A, Fisher Scientific.

Autosampler vial insert, 500 µL, catalog number 03-375-3B, Fisher Scientific.

Vial, 11-dram (45 mL), screw thread, catalog number 60958A-11, Kimble Chase.

Vial cap, 11-dram (45 mL), White Polypropylene PE Foam/PTFE, catalog number 02-912-069, Fisher Scientific.

Vial, 8-dram, amber glass, catalog number 03-339-23E, Fisher Scientific.

Cap, Teflon-lined screw-cap to fit 8-dram vial, part number 02-913-115, Fisher Scientific.

Syringe, 3-mL, luer-lok, BD disposable, part number 14-823-435, Fisher Scientific.

Volumetric flask, 10 mL, 20 mL, and 100 mL, Fisher Scientific.

6. Reagents

Acetonitrile, Optima, catalog number A996, ThermoFisher Scientific.

Ammonium Acetate, HPLC grade, catalog number A639, Fisher Scientific.

Ammonium Hydroxide, Certified A.C.S. Plus, 29.7%, catalog number A669, Fisher Scientific.

Ethyl Acetate, Optima, catalog number. E196, Fisher Scientific.

Ethyl Alcohol, Absolute, 200 proof, ACS Reagent, catalog number 61509, ACROS Organics.

Ethyl Chloroformate, 97%, catalog number 185892, Sigma Aldrich.

Formic Acid, Optima LC/MS Grade, catalog number A117, ThermoFisher Scientific.

Formic Acid, 98+%, catalog number 14793, ACROS Organics.

Glycerol, Reagent ACS, catalog number 41098, ACROS Organics.

Hydrochloric Acid, Certified A.C.S. Plus, catalog number A144, Fisher Scientific.

Isopropanol, HPLC, A.C.S., catalog number A451, Fisher Scientific.

Methanol, Optima, A.C.S., catalog number A454, ThermoFisher Scientific.

Pyridine, HPLC grade 99.5+%, catalog number 22905, AlfaAesar.

Sodium hydroxide 10N (30% W/W), catalog number SS255, ThermoFisher Scientific.

Trifluoroacetic Acid, Optima LCMS grade, catalog number A116, ThermoFisher Scientific.

Water, HPLC grade, W5, ThermoFisher Scientific.

Water, Optima-LCMS grade, catalog number W6, ThermoFisher Scientific.

7. 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.

Methanol/10N sodium hydroxide (100/1, v/v)

Measure 500 mL of methanol with a graduated cylinder and transfer to a bottle. Measure 5 mL of 10N sodium hydroxide with a serological pipet and transfer into the same bottle. Mix thoroughly.

Hydrochloric acid, 0.2N

Measure 160 mL of HPLC grade water using a graduated cylinder and transfer to a bottle. Measure 40 mL of 1N hydrochloric acid using a graduated cylinder. Carefully transfer the measured hydrochloric acid into the bottle containing 160 mL water. Mix thoroughly.

Hydrochloric acid, 1N

Partially fill a 250-mL graduated mixing cylinder with HPLC grade water. Measure 20.675 mL of hydrochloric acid, using a pipet, and carefully add to the measured HPLC grade water contained in the mixing cylinder. Bring to a final volume of 250 mL with HPLC grade water and mix thoroughly.

Acetonitrile/ 1N hydrochloric acid (15/85, v/v)

Measure 30 mL of acetonitrile with a graduated cylinder and transfer to a bottle. Add 170 mL of 1N hydrochloric acid solution measured with a graduated cylinder into the bottle. Mix thoroughly.

20 mM Ammonium Acetate

Weigh 1.54 g of ammonium acetate and transfer to a plastic bottle. Measure 1000 mL of HPLC grade water with a graduated cylinder and transfer to the bottle. Mix thoroughly to solubilize ammonium acetate. Store in the refrigerator at 2-8 °C when not in use. Discard after 14 days.

20 mM Ammonium Acetate/Methanol (70/30, v/v)

Measure 75 mL of methanol with a graduated cylinder and transfer to a bottle. Measure 175 mL of 20 mM ammonium acetate with a graduated cylinder and transfer to the bottle. Mix thoroughly. Store in refrigerator at 2-8 °C when not in use.

20% Ammonium hydroxide

Measure 32 mL of HPLC grade water with a graduated cylinder and transfer to a bottle. Measure 70 mL of the 29.7% ammonium hydroxide solution with a graduated cylinder and carefully transfer to the bottle. Mix thoroughly.

Ethyl acetate/Trifluoroacetic acid (98/2, v/v)

Measure 490 mL of ethyl acetate with a graduated cylinder and transfer to a bottle. Pipette 10 mL of trifluoroacetic acid in to the bottle. Mix thoroughly.

Ethanol/Glycerol (90/10, v/w)

Weigh 10.00 g of glycerol into a bottle. Measure 90 mL of ethanol with a graduated cylinder and transfer to the bottle. Mix thoroughly.

Acetonitrile/pyridine/ethanol (4/2/1, v/v/v)

Pipet 16 mL of acetonitrile to a bottle. Using a pipet, add 8 mL of pyridine and 4 mL of ethanol into the bottle. Mix thoroughly. Discard after 7 days.

Acetonitrile/ethyl chloroformate (9/1, v/v)

Pipet 18 mL of acetonitrile into a bottle. 2 mL of ethyl chloroformate were added to the bottle using a syringe. Mix thoroughly. Store in refrigerator at 2-8 °C. Discard after 7 days.

Acetonitrile/water/formic acid (50/50/0.5%, v/v/v)

Measure 100 mL of acetonitrile with a graduated cylinder and transfer to a bottle. Measure 100 mL of HPLC grade water with a graduated cylinder and transfer into the bottle. Pipette 1 mL of formic acid into the bottle. Mix thoroughly.

Water with 0.1% formic acid

Measure 2000 mL of HPLC grade water with a graduated cylinder and transfer to a bottle. Pipette 2 mL of formic acid into the bottle. Mix thoroughly.

Acetonitrile with 0.1% formic acid

Measure 2000 mL of HPLC grade acetonitrile with a graduated cylinder and transfer to a bottle. Pipette 2 ml of formic acid into the bottle. Mix thoroughly.

Isopropanol/Methanol/water (2/2/1, v/v/v)

Transfer 4000 mL of isopropyl alcohol and 4000 mL of methanol to a carboy and, using a graduated cylinder, add 2000 mL of water. Mix thoroughly.

Methanol/Acetonitrile/water (1/1/2, v/v/v)

Transfer 8000 mL of water to a carboy, and add 4000 mL each of methanol and acetonitrile. Mix thoroughly.

8. Preparation of Spiking Solutions

0.02006 g and 0.02008 g of the picloram analytical standard was weighed and diluted with methanol to obtain a 1-mg/mL (1,000-µg/mL) picloram stock solution.

2.00 mL of the 1-mg/mL picloram solution was pipetted into a 20-mL volumetric flask and diluted to volume with methanol to obtain a 100-µg/mL picloram spiking solution.

0.200 mL of the 1-mg/mL picloram spiking solution was pipetted into a 20-mL volumetric flask and diluted to volume with methanol to obtain a 10.0-µg/mL picloram spiking solution.

2.00 mL of the 10.0-µg/mL picloram spiking solution was pipetted into a 20-mL volumetric flask and diluted to volume with methanol to obtain a 1.00-µg/mL picloram spiking solution.

2.00 mL of the 1.00-µg/mL picloram spiking solution was pipetted into a 20-mL volumetric

flask and diluted to volume with methanol to obtain a 0.100-µg/mL picloram spiking solution.

2.00 mL of the 0.100-µg/mL picloram spiking solution was pipetted into a 20-mL volumetric flask and diluted to volume with methanol to obtain a 0.0100-µg/mL picloram spiking solution.

2.00 mL of the 0.0100-µg/mL picloram spiking solution was pipetted into a 20-mL volumetric flask and diluted to volume with methanol to obtain a 0.001000-µg/mL picloram spiking solution.

9. Preparation of Internal Standard

0.00198 g of $^{13}C_2{}^{15}N$-picloram internal standard was accurately weighed and diluted with methanol to obtain a 99-µg/mL stock solution of $^{13}C_2{}^{15}N$-picloram internal standard.

202 µL of the 99-µg/mL $^{13}C_2{}^{15}N$-picloram internal standard solution was pipetted into a 20-mL volumetric flask and diluted to volume with methanol to obtain a 1.0-µg/mL $^{13}C_2{}^{15}N$-picloram internal standard solution.

2.00 mL of the 1.0-µg/mL $^{13}C_2{}^{15}N$-picloram internal standard solution was pipetted into a 20-mL volumetric flask and diluted to volume with methanol to obtain a 0.10-µg/mL $^{13}C_2{}^{15}N$-picloram internal standard solution.

2.00 mL of the 0.10-µg/mL $^{13}C_2{}^{15}N$-picloram internal standard solution was pipetted into a 20-mL volumetric flask and diluted to volume with methanol to obtain a 0.010-µg/mL $^{13}C_2{}^{15}N$-picloram internal standard solution.

10. Preparation of Calibration Solutions

Aliquots of spiking solutions were dispensed into a series of 20-mL volumetric flasks as indicated in the following table, and diluted to volume with methanol:

Concentration of Spiking Solution (µg/mL)	Aliquot of Spiking Solution (µL)	Final Solution Volume (mL)	Intermediate Calibration Solution Conc. (ng/mL[a])	Calibration Solution Final Conc. (ng/mL[b])	Equivalent Sample Concentration (ng/g[c])
0.100	150	20	0.75	0.150	0.3
0.100	500	20	2.5	0.500	1
1.00	100	20	5.0	1.00	2
1.00	250	20	12.5	2.50	5
1.00	500	20	25	5.00	10
10.0	250	20	125	25.0	50
10.0	500	20	250	50.0	100

[a]Concentration after diluting to volume with methanol
[b]Concentration after taking a 100-µL aliquot of intermediate calibration solution, evaporation, and derivatization
[c]The equivalent sample concentration is based on fortifying a 1-gram sample, extracting with 20 mL, taking a 5-mL aliquot, and reconstituting in 0.5 mL.

All standard solutions and sample extracts are stored refrigerated at 2-8 °C in an 8-dram amber vial.

11. Sample Analysis

11.1. Weigh 1.0 ± 0.01 g of the sample into a 50-mL centrifuge tube
(Note: Add additional 3 unfortified control samples for matrix-matched calibration standards.)

11.2. For recovery samples, add appropriate aliquots of the spiking solution to obtain concentrations ranging from 0.3 – 1,000,000 ng/g. (**Note**: Allow samples to sit for ~5 minutes). For example, see table below:

Concentration of Fortified Sample (ng/g)	Concentration of Spiking Solution (µg/mL)	Volume of Spiking Solution (µL)	Dilution Factor	Equivalent Concentration (ng/mL)
0.3	0.00100	300	1	0.15
1.0	0.0100	100	1	0.5
10	0.100	100	1	5.0
100	1.00	100	50	1.0
1,000,000	10,000	100	50,000	10

11.3. Add 20 mL of methanol:10 N sodium hydroxide (100:1, v:v) extraction solution to the sample bottle and seal tightly with a cap. Vortex to mix.

11.4. Shake the sample for a minimum of 60 minutes on a reciprocating shaker set at approximately 280 excursions/minute.

11.5. Allow the samples to stand at room temperature overnight (minimum of 12 hours). (Note: For grass compost samples (typically brown in color) that have been aged for 45 days or longer, use the Branson sonifier to sonicate the samples for 10 minutes at 80% amplitude using pulse on for 20 seconds and off for 5 seconds. Then, let the samples stand at room temperature overnight.)

11.6. The next day, centrifuge the samples at ~3,000 rpm for ~5 minutes.

11.7. Carefully decant the supernatant to a new centrifuge tube; avoid transfer of particulates as much as possible. (Acceptable stopping point; store extracts in refrigerator.)

11.8. For samples with concentrations lower than or equal to 75 ng/g, continue to step 11.9.
For samples with a concentration greater than 75 ng/g, continue to step 11.18.

11.9. For samples with concentrations lower than or equal to 75 ng/g, transfer 5 mL of the extract into 11-dram (45-mL) vials. (Note: additional unfortified control aliquots should be taken for matrix-matched standard preparation.)

11.10. Add 100 µL of the 0.01-µg/mL internal standard solution to the 5 mL of extract. (Note: Do not add internal standard at this step to the aliquots that will be used for matrix-matched standard preparation.)

11.11. Evaporate the extract to dryness on a N-Evap set at ~40 °C using a gentle stream of nitrogen (about 1.0 L/min).

11.12. Reconstitute by adding 3 mL of 0.2N HCl. Vortex.

11.13. Add 2 mL of water to each vial. Vortex.

11.14. Purify the samples using the following HLB SPE procedure:

11.14.1. Condition HLB cartridges with 3 mL of methanol, followed by 3 mL of 0.2N HCl. [**Note**: The drip rate for each solvent/solution should be ~2 drop/second (low vacuum may be used if needed)]. Pull an ~5-second vacuum at the end of the conditioning. Discard the eluate.

11.14.2. Load samples onto the HLB cartridges. [**Note**: The drip rate should be ~1 drop/second (low vacuum may be used if needed)]. Pull an ~5-second vacuum at the end of the loading. Discard the eluate.

11.14.3. Rinse each vial with 5 mL of 15:85 acetonitrile:1N HCl and load onto the respective HLB cartridge. [**Note**: The drip rate should be ~1 drop/second (low vacuum may be used if needed)]. Dry the cartridge for ~2-3 minutes. Discard the eluate.

11.14.4. Elute the cartridges with two 3-mL aliquots of the 70:30 20 mM ammonium acetate:methanol solution. Collect this elution in 16 x 125 mm vials with screw neck top. (**Note**: The drip rate should be ~1 drop/second, low vacuum may be used if needed).

11.14.5. Evaporate samples on a Turbovap set at ~40 °C using a gentle stream of nitrogen to ensure that all of the organic is removed (about 1.0 L/min). **Approximately 4 mL of solution should remain.** Use a pH strip to check the pH of each control sample and adjust to pH of 5-7 using the 20% ammonium hydroxide solution or 1N HCl as necessary (add in approx. 10-µL increments). Based on the adjustments made to the control, adjust the pH for each sample of the same matrix accordingly. **It is important to adjust the pH prior to AFFINIMIP step.**

11.15. Purify the samples using the following AFFINIMIP procedure.
(**Note: Do not pull a vacuum unless directed by the procedure)**

11.15.1. Condition the cartridge with 6 mL of acetonitrile. Discard the eluate.

11.15.2. Equilibrate the cartridge with 6 mL of water. Discard the eluate.

11.15.3. Load the sample to the SPE cartridge. Discard the eluate.

11.15.4. Add 3 mL of water to the sample vial, vortex and transfer this to the respective cartridge. Discard the eluate.

11.15.5. Wash the cartridge with two 3-mL aliquots of water. Discard the eluate. Dry the SPE cartridge for ~2 minutes under vacuum after the final aliquot.

11.15.6. Wash the cartridge with two 3-mL aliquots of acetonitrile. Discard the eluate. Dry the SPE cartridge for ~2 minutes under vacuum after the final aliquot.

11.15.7. Elute with three 4-mL aliquots of ethyl acetate:trifluoroacetic acid (98:2, v:v) into a 16 x 125 mm vial containing 20 µL of 10:90 glycerol:ethanol solution.

11.16. 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 1.5 L/min; the samples should be dry in approximately 60-70 minutes. For the additional unfortified control aliquots to be used for matrix-matched standards, continue to step 11.17. For other samples, continue to step 11.20.

11.17. For matrix matched calibration standards, follow the directions below:

11.17.1. Aliquot 100 µL of each intermediate calibration solution into separate vials of the additional unfortified controls (from step 11.16).
(Note: the unfortified control aliquots should not have had internal standard added at step 11.10.)

11.17.2. Add 100 µL of the 0.01-µg/mL internal standard solution. Vortex to mix.

11.17.3. Evaporate to dryness on a Turbovap set at 40 °C with a gentle stream of nitrogen (about 1.0 L/min).

11.18. For all samples with concentrations greater than 75 ng/g, follow the dilution procedures below:

11.18.1. Pipet 1.0 mL of sample extract from step 11.8 into a vial and add 1.0 mL of 0.1N HCl. Vortex to mix.

11.18.2. Perform the appropriate dilution(s) following the scheme in the table below for each sample starting with the acidified extract from step 11.18.1. Vortex to mix the sample after each methanol dilution.

Conc of Fortified Sample (ng/g)	Dilution Factor	Aliquot of Acidified Extract (mL)	Methanol to be added to Acidified Aliquot (mL)	Aliquot of 50X (mL)	Methanol to be added to 50X Aliquot (mL)	Aliquot of 500X (mL)	Methanol to be added to 500X Aliquot (mL)	Aliquot of 5,000X (mL)	Equiv. Conc. (ng/mL)
75-1,000	50	0.2	-	-	-	-	-	-	0.75-10.0
1,000-10,000	500	0.2	1.8	0.2	-	-	-	-	1.0-10.0
10,000-100,000	5,000	0.2	1.8	0.2	1.8	0.2	-	-	1.0-10.0
100,000-1,000,000	50,000	0.2	1.8	0.2	1.8	0.2	1.8	0.2	1.0-10.0

11.18.3. Pipet 100 µL of the 0.01-µg/mL internal standard solution and 20 µL of the 90:10 ethanol:glycerol solution to the 0.2 mL of the final diluted extract. Vortex to mix.

11.18.4. Evaporate the extract to dryness on a Turbovap set at 40 °C using a gentle stream of nitrogen (about 1.0 L/min). Continue to step 11.20.

11.19. For neat calibration standards, aliquot 100 µL of each intermediate calibration solution into separate 16 x 125 mm vials. Add 100 µL of the 0.01-µg/mL internal standard solution. Add 20 µL of the 10:90 glycerol:ethanol solution. Evaporate to dryness on a Turbovap set at ~40 °C using a gentle stream of nitrogen (about 1.0 L/min).

11.20. Add 200 µL of the acetonitrile:pyridine:ethanol (4:2:1, v:v:v) solution, to all vials. Vortex to mix. Sonicate if necessary to dissolve.

11.21. Derivatize the samples and the calibration standards by pipetting 200 µL of the acetonitrile:ethyl chloroformate (9:1, v:v) solution into the tubes.

11.22. Immediately vortex the samples and the standards for a few seconds. Then allow the mixture to react at room temperature for at least 5 minutes.

11.23. Add 100 µL of the 50:50 water:acetonitrile solution containing 0.5% formic acid. Vortex to mix.

11.24. Transfer samples to glass autosampler vials.

11.25. Analyze the derivatized calibration standards and samples by HPLC with positive-ion electrospray tandem mass spectrometry. (Acceptable stopping point if sample is kept refrigerated.)

11.26. Determine the suitability of the chromatography system using the following performance criteria:

11.26.1. 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.

11.26.2. Appearance of chromatograms: Visually determine the chromatograms 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 picloram at the 0.5 ng/mL calibration standard.

11.26.3. Re-analyze samples which contain concentrations of picloram greater than 80% of the highest standard starting with the extract from step 11.7 and performing the appropriate dilution(s) as indicated in step 11.18. The gross analyte concentration should be at least 30% above the lowest calibration standard and at least 20% less than the highest calibration standard.

12. Supplemental 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 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.

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

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

12.4. Neat calibration standards should be used to calculate the results for the reagent blanks and the samples prepared following step 11.18 (for samples with a picloram concentration greater than 75 ng/g). Matrix matched standards should be used to calculate the results for samples prepared following steps 11.9-11.17 (for untreated control samples and samples with a picloram concentration less than or equal to 75 ng/g).

13. Instrumental Conditions

Typical HPLC Operating Conditions

Instrumentation	HPLC System: Agilent 1290 Series
Column:	Waters HSS T3
	2.1 x 100 mm, 1.8 µm
In-line filter:	KrudKatcher Ultra (0.5 µm depth filter x 0.004 in ID)
Column Temperature:	15 °C
Autosampler Temperature:	15 °C
Injection Volume:	5 µL
Injection Wash:	Strong Wash: 2/2/1 Isopropanol/Methanol/Water
	Weak Wash: 1/1/2 Methanol/Acetonitrile/Water
Mobile Phase:	A – Water with 0.1% formic acid
	B – Acetonitrile with 0.1% formic acid
Run Time:	21.7 min

Gradient:	Time	Flow	Solvent B
	(min)	(mL/min)	(percent)
	0.0	0.3	5
	2.0	0.3	40
	9.0	0.3	46
	14.6	0.3	95
	15.6	0.3	95
	16.6	0.6	5
	20.7	0.6	5
	21.7	0.3	5
Diverter Program			
Flow to Waste	0.0 min → 6.00 min		
Flow to Source	6.00 min → 10.5 min		
Flow to Waste	10.5 min → 21.7 min		

Typical Mass Spectrometry Operating Conditions

Instrumentation:	QTRAP 5500 MS System				
	AB SCIEX Analyst version 1.6.2 data system				
Ionization :	Electrospray				
Mode:	Multiple Reaction Monitoring (MRM)				
Ion Polarity:	positive				
Scan Type:	MRM				
Resolution:	Q1 – unit, Q3 – unit				
Curtain Gas (CUR)	40 psi				
Collision Gas	High				
IonSpray Voltage	5500 V				
Temperature	400 °C				
Ion Source Gas 1	50 psi				
Ion Source Gas 2	50 psi				
Entrance Potential	10 V				
Declustering	110 V				
Analytes	Precursor Ion, Q1 *m/z*	Product Ion Q3 *m/z*	Dwell Time msec	Collision Energy V	Cell Exit Potential V
Picloram (269/168)	269	168	200	46	10
Picloram (271/143)	271	143	200	65	10
Picloram IS	274	144	200	65	10