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

INTRODUCTION

Scope

This method is applicable for the quantitative determination of residues of clopyralid in compost. The method was validated over the concentration range of 0.700 – 40.0 ng/g with a validated limit of quantitation of 0.700 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.

This study was conducted under laboratory study 191812.

Method Principle

Residues of clopyralid are extracted from the sample matrices by shaking with 0.1N NaOH in methanol. After subsequent sonication, the extracts sit overnight at room temperature. The next day, after centrifugation, an aliquot of each extract is transferred and dried. The samples are reconstituted with 1 N HCl and then undergo solid phase extraction. After drying the SPE eluent, internal standard (IS) is added, and samples are dried again. Samples are derivatized, nearly dried, and then reconstituted for analysis. The final reconstituted extracts were analyzed for derivatized clopyralid using two structurally characteristic MS/MS transitions by liquid chromatography with tandem mass spectrometry (LC-MS/MS).

Test Substance/Reference Compounds/Analytical Standards

Test Substance	TSN	Percent Purity	Recertification Date	Reference
Clopyralid	TSN301194	99.9%	31-Jul-2020	FAPC16-000216

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

Test Substance	TSN	Percent Purity	Recertification Date	Reference
X12566799	TSN311080	99%	18-Jul-2022	FAPC17-000376

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 an analytically relevant concentration and if observed, should be addressed for accurate determination of analyte concentrations.

To determine the contribution of the unlabeled clopyralid to the clopyralid internal standard, a sample spiked with clopyralid 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 clopyralid internal standard to the unlabeled clopyralid, a sample was spiked at the working concentration of internal standard in neat solution that was not spiked with clopyralid. 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. The results are summarized in Table 2.

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.

Clopyralid	*m/z* Q1/Q3 248/110 (quantitative) *m/z* Q1/Q3 250/112 (confirmatory)
Clopyralid IS	*m/z* Q1/Q3 253/115

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. Refer to Figures 3-4 for example calibration plots and to Figure 5 for an example calculation.

Confirmation of Residue Identity

The method is specific for the determination of clopyralid 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.

More decimal places than are shown in tables were used to calculate values presented in this report. Therefore, minor differences due to rounding may be found when calculating values from data in tables presented here.

REFERENCES

1. *OCSPP 850.6100: Environmental Chemistry Methods and Associated Independent Laboratory Validation*; United States Environmental Protection Agency, U.S. Government Printing Office: Washington, DC, 2012; EPA-712-C-001.

2. SANCO/3029/99 rev.4 (11 July 2000), *Guidance for Generating and Reporting Methods of Analysis in Support of Pre-Registration Data Requirements for Annex II (Part A, Section 4) and Annex III (Part A, Section 5) of Directive 91/414*; Directorate General Health and Consumer Protection, European Commission.

3. SANCO/825/00 rev. 8.1 (16 November 2010), *Guidance Document on Pesticide Residue Analytical Methods*; Directorate General Health and Consumer Protection, European Commission.

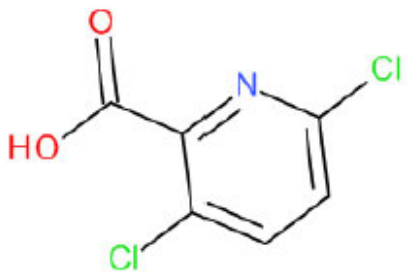
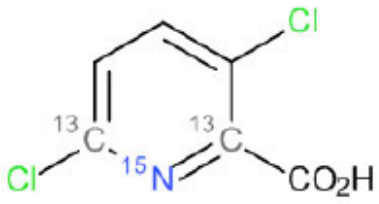
4. *Residue Chemistry Guidelines,* Regulatory Directive Dir98-02, Section 3, *Residue Analytical Method*, Submission Management and Information Division, Pest Management Regulatory Agency, Health Canada: Ottawa, Ontario, June 1, **1998**.

5. Baldwin, R.; Bethem, R. A.; Boyd, R. K.; Budde, W. L.; Cairns, T.; Gibbons, R. D.; Henion, J. D.; Kaiser, M. A.; Lewis, D. L.; Matusik, J. E.; Sphon, J. A.; Stephany, R. W.; Trubey, R. K. *J. Am. Soc. Mass Spectrom.* **1997**, *8*, 1180-1190.

6. Hall, L.R., "^{14}C-Clopyralid: Metabolism in Confined Rotational Crops with a 30-Day Plant-back Interval", Study 130733, 2018, unpublished report of Dow AgroSciences, LLC.

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

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

Identifying Information	Structure
Common Name of Compound: Clopyralid Molecular Formula: $C_6H_3Cl_2NO_2$ Formula Weight: 192.00 CAS Number: 1702-17-6	
Common Name of Compound: Clopyralid IS Molecular Formula: $C_4{}^{13}C_2H_3Cl_2{}^{15}NO_2$ Formula Weight: 194.97 CAS Number: NA	

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

Scope

This method is applicable for the quantitative determination of Clopyralid in compost. This method is applicable over a concentration range 0.700 – 40.0 ng/g.

Principle

Residues of clopyralid are extracted from the sample matrices by shaking with 0.1N NaOH in methanol. After subsequent sonication, the extracts sit overnight at room temperature. The next day, after centrifugation, an aliquot of each extract is transferred and dried. The samples are reconstituted with 1 N HCl and then undergo solid phase extraction. After drying the SPE eluent, internal standard (IS) is added, and samples are dried again. 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.

Digital sonifier, Model 450, Branson Ultrasonics Corporation.

Evaporator, TurboVap LV, model number 415000, Biotage.

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.

Positive Pressure Manifold, System 48. Cerex.

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.

Ultrasonic Bath, 2.8L, Model 15337433, Fisher Scientific.

Vacuum manifold, Model spe-12G, Mallinckrodt Baker, Inc.

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

Chromatographic System

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

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

Liquid chromatograph, 1290 Infinity I or II Series, Agilent.

Mass spectrometer, Model QTRAP 5500, AB SCIEX.

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

Glassware and Materials

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

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.

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.

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

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

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, 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, 11-dram, screw thread, catalog number 60958A-11, Kimble Chase.

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.

Volumetric flask, 50 mL, catalog number K623010-0050, Fisher Scientific.

Volumetric flask, 100 mL, catalog number K623010-0100, Fisher Scientific.

Reagents

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

Ethyl Acetate, HPLC grade 99%, catalog number. E195-4, Fisher Scientific.

1-Butanol, HPLC grade 99%, catalog number. A383-1, Fisher Scientific.

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

Formic Acid, 99% purity, catalog number 27048-0010, ACROS Organics.

Glycerol, ACS grade, catalog number G33-500, Fisher Scientific.

Hydrochloric Acid, 0.1N, catalog number SA54-1, Fisher Scientific.

Hydrochloric Acid, 1N, catalog number SA48-500, Fisher Scientific.

Isopropanol, Optima, ACS 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 10N, catalog number 7470-1, RICCA Chemical.

Trifluoroacetic Acid, HPLC/Spectro grade, catalog number 28901, Thermo 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.

Methanol/10N Sodium Hydroxide (100/1, v/v)

Measure 4000 mL of methanol with a graduated cylinder and transfer to a bottle. Measure 40 mL of 10N sodium hydroxide with a graduated cylinder and transfer into the same bottle. Mix thoroughly.

Hydrochloric Acid, 0.1N (May be purchased commercially)

Measure 900 mL of HPLC grade water using a graduated cylinder and transfer to a bottle. Measure 100 mL of 1N hydrochloric acid using a graduated cylinder. Carefully transfer the measured hydrochloric acid into the bottle containing 900 mL water. Swirl the bottle to 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.

Acetonitrile/pyridine/1-butanol (22/2/1, v/v/v)

Add 88 mL of acetonitrile to a bottle. Using a pipette, add 8 mL of pyridine and 4 mL of 1-butanol into the bottle. Mix thoroughly. Discard after 21 days.

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

Pipette 18 mL of acetonitrile into a bottle. Pipet 2 mL of butyl chloroformate into the bottle. Mix thoroughly. Store in refrigerator at 4°C. Discard after 21 days.

Water with 0.1% formic acid (May be purchased commercially)

Pipette 4.0 mL of formic acid into a bottle containing 4000 mL of water. Mix thoroughly.

Acetonitrile with 0.1% formic acid (May be purchased commercially)

Pipette 4.0 mL of formic acid into a bottle containing 4000 mL of acetonitrile. Mix thoroughly.

Methanol/acetonitrile/isopropanol/water (30/30/30/10, v/v/v/v)

Measure 1200 mL of isopropyl alcohol with a graduated cylinder and transfer to a bottle. Measure with a graduated cylinder 1200 mL of methanol, 1200 mL of acetonitrile and 400 mL of water and transfer into the bottle. Mix thoroughly.

Seal Wash (water/isopropanol, 90/10, v/v)

Combine 3600 mL of water with 400 mL of isopropanol. Mix thoroughly.

Preparation of Stock Solutions

The specific masses and volumes indicated below need not be used as long as the final concentrations for required solutions are accurately prepared and documented. Not all of the individual stock solutions may be required.

Accurately weigh approximately 0.007 g of clopyralid analytical standard and dilute to volume with an appropriate amount of methanol to obtain a 10.0 mg/mL clopyralid stock solution.

Pipette 2.00 mL of the 10.0 mg/mL clopyralid stock solution and dilute to 20.0 mL with methanol to obtain a 1.00 mg/mL clopyralid stock solution.

Pipette 2.00 mL of the 1.00 mg/mL clopyralid stock solution and dilute to 20.0 mL with methanol to obtain a 100 µg/mL clopyralid stock solution.

Pipette 2.00 mL of the 100 µg/mL clopyralid stock solution and dilute to 20.0 mL with methanol to obtain a 10,000 ng/mL clopyralid stock solution.

Pipette 2.00 mL of the 10,000 ng/mL clopyralid stock solution and dilute to 20.0 mL with methanol to obtain a 1000 ng/mL clopyralid stock solution.

Pipette 2.00 mL of the 1000 ng/mL clopyralid stock solution and dilute to 20.0 mL with methanol to obtain a 100 ng/mL clopyralid stock solution.

Pipette 1.00 mL of the 100 ng/mL clopyralid stock solution and dilute to 20.0 mL with methanol to obtain a 5.00 ng/mL clopyralid stock solution.

Preparation of Intermediate Calibration STD Spiking Solutions

Prepare the following Intermediate Calibration STD Spiking Solutions in methanol:

Concentration of Stock Solution (ng/mL)	Aliquot of Stock Solution (µL)	Final Solution Volume (mL)	Intermediate Calibration STD Solution Concentration (ng/mL)	Intermediate Calibration STD Spiking Solution	*Equivalent* Neat Cal STD Concentration (ng/g)*
100	210	20.0	1.05	A	0.210
100	400	20.0	2.00	B	0.400
100	700	20.0	3.50	C	0.700
100	1000	20.0	5.00	D	1.00
100	2000	20.0	10.0	E	2.00
1000	500	20.0	25.0	F	5.00
1000	1000	20.0	50.0	G	10.0
1000	2000	20.0	100	H	20.0
10,000	300	20.0	150	I	30.0
10,000	500	20.0	250	J	50.0

*Note: Equivalent Sample Conc (ng/g) is based on 1 g of sample diluted in 20 mL, aliquot factor of 10.00 mL (10/20 of extracted sample). Only 100 µL of STDs A – J are used to prepare neat calibration standards. Overall method factor is 0.500.

Preparation of Internal Standard

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

Combine 100 µL of the 100 µg/mL $^{13}C_2{}^{15}N$-clopyralid internal standard solution with 19.9 mL methanol to obtain a 500 ng/mL $^{13}C_2{}^{15}N$-clopyralid internal standard solution. Mix well.

Pipette 5.00 mL of the 500 ng/mL $^{13}C_2{}^{15}N$-clopyralid internal standard solution into a 50.0-mL volumetric flask and dilute to volume with methanol to obtain a 50.0 ng/mL $^{13}C_2{}^{15}N$-clopyralid internal standard solution. Mix well.

Analysis Procedure

For procedural recovery samples:

1. For reagent blank, add 20 mL of 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, add aliquots of the spiking solution to obtain concentrations corresponding to the table below:

Fortified Sample	Volume of Stock Solution (µL)	Concentration of Stock Solution (ng/mL)	Fortification Level (ng/g)
LOD	42.0	5.00	0.210
LOQ	140.0	5.00	0.700
10x LOQ	70.0	100	7.00
57x LOQ	40.0	1000	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 mL of methanol/10 N sodium hydroxide (100/1, v/v) extraction solution to each 50-mL centrifuge tube and seal tightly with a cap. Vortex to mix.
6. Shake the sample for a minimum of 60 minutes on a reciprocating shaker set at approximately 240 excursions/minute.
7. Use the Branson sonifier to sonicate the samples for 10 minutes at 70-80% amplitude using pulse on for 20 seconds and off for 5 seconds.
8. Allow the samples to stand at room temperature overnight (minimum of 12 hours).
9. The next day, centrifuge the samples at 3,000 rpm for 5 min.
10. Pipette 10.0 mL of each extract supernatant into labeled 45 mL glass tubes.
11. Dry all samples to near dryness on a Turbovap set at 40°C using a gentle stream of nitrogen (about 1.0 to 1.2 L/min). Absolute dryness is not necessary.
12. Reconstitute with 4.00 mL of 1N HCl. Mix well.

13. 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. Load each sample onto the SPE cartridge. Aim for an elution rate of about 1-2 seconds between drops. Discard the eluate.
    d. Rinse each corresponding 45 mL glass tube with 3 mL of water, and use to wash the corresponding SPE cartridge. Aim for an elution rate of about 1-2 seconds between drops. Discard the eluate.
    e. Repeat the previous step (d) with an additional 3 mL water. Discard the eluate.
    f. 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.
    g. Elute with two 4.00 mL aliquots of ethyl acetate/trifluoroacetic acid (98/2, v/v) into a 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.
14. 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 15. For all other samples, skip to step 16.
15. For neat calibration standards, aliquot 100 µL of each Intermediate Calibration STD Spiking Solution (A – J) into separate, labeled, clean test tubes.
16. To all samples, add 100 µL of the 50.0 ng/mL internal standard solution. Vortex to mix.
17. 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>all</u> the methanol added in the previous step(s) be completely dried off before adding the derivatizing agents in the next two steps.]**
18. 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.
19. Derivatize the samples and the calibration standards by pipetting 100 µL of the acetonitrile/butyl chloroformate (9/1, v/v) solution into the tubes.
20. Immediately vortex the samples and the standards for a few seconds. Then allow the mixture to react at room temperature for about 15 minutes.

21. Add 250 µL 0.1% formic acid in water to each sample and mix well. Sonicate about 30 seconds.

22. 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. Sometimes, especially for pasture grass compost, additional filtering may be necessary.

23. Analyze the derivatized calibration standards and samples by HPLC with positive-ion electrospray tandem mass spectrometry. Glass vials are preferable to plastic vials.

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 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:	40 °C		
Autosampler Temperature:	10 °C		
Injection Volume:	20 µL		
Injection Wash:	30/30/30/10 Methanol/isopropanol/acetonitrile/water 20 seconds at flush port		
Seal Wash:	90/10 Water/isopropanol		
Mobile Phase:	A – Water with 0.1% formic acid		
	B – Acetonitrile with 0.1% formic acid		
Run Time:	approximately 6.5 minutes		
Flow Rate:	600 µL/min		
Gradient:	Time	Solvent A	Solvent B
	(min)	(percent)	(percent)
	0.0	45	55
	2.0	45	55
	3.5	37	63
	4.0	5	95
	5.0	5	95
	5.5	45	55
	6.5	45	55
Diverter Program			
Flow to Waste	0.0 min → 2.5 min		
Flow to Source	2.5 min → 3.5 min		
Flow to Waste	3.5 min → end of run		

Typical Mass Spectrometry Operating Conditions

Instrumentation:	QTRAP 5500 MS System				
	AB SCIEX Analyst version 1.6.3 data system				
Ionization :	Electrospray				
Mode:	Multiple Reaction Monitoring (MRM)				
Ion Polarity:	positive				
Scan Type:	MRM				
Resolution:	Q1 – unit, Q3 – unit				
Curtain Gas (CUR)	20 psi				
Collision Gas (CAD):	10				
IonSpray Voltage (IS):	5500 volts				
Temperature (TEM):	600 °C				
Ion Source Gas 1 (GS1)	45 psi				
Ion Source Gas 2 (GS2)	55 psi				
Entrance Potential (EP):	10 volts				
Declustering Potential:	100 V				
Analytes	Precursor Ion, Q1 m/z	Product Ion Q3 m/z	Dwell Time msec	Collision Energy V	Cell Exit Potential V
Clopyralid (248/110)	248	110	70	60	14
Clopyralid (250/112)	250	112	70	60	14
Clopyralid IS (253/115)	253	115	70	60	14

Clopyralid transition 248/110 is used for quantitation. Clopyralid transition 250/112 is used for confirmation.

Note: An interference from the Oasis MAX SPE cartridge arises for transition 248/174 (Collision Energy 20V), which is why it was not selected as the confirmation transition. Use of alternate transitions corresponding to the other viable chlorine isotope precursors (250/112 and 250/176) suffer from interferences arising only in samples containing the $^{13}C_2{}^{15}N$-clopyralid internal standard. The amount of IS added was minimized in order to also reduce this interference in the 250/112 transition, the selected confirmation transition.