Independent Laboratory Validation of Dow AgroSciences Method 120611, "Method Validation Study for the Determination of Residues of Clopyralid and Picloram in Drinking Water, Ground Water, and Surface Water by LC-MS/MS"

INTRODUCTION

Scope

The objective of this study was to assess and to independently validate the method described in the Dow AgroSciences Method 120611, "Method Validation Study for the Determination of Residues of Clopyralid and Picloram in Drinking Water, Ground Water, and Surface Water by LC-MS/MS". The methodology was successfully independently validated over the concentration range of 0.05 - 0.5 µg/L with a verification of the limit of quantification of 0.050 µg/L.

Three different water matrices were used for validation. These were drinking water, ground water and surface water samples.

Chemical names, molecular structures, molecular formulas and molecular weights for the analytes are given in Table 1.

The independent laboratory, the Study Director, and the analyst chosen to conduct the ILV were unfamiliar with the method.

This study was conducted to fulfill data requirements outlined in EC Regulation No. 1107/2009 (1), Guidance document on pesticide residue analytical methods, SANCO/825/00 rev. 8.1 (2), EPA PR Notice 96-1 (3), EPA PR Notice 2011-3 (4) for reporting, EPA Guideline; OCSPP 850.6100 (5).

Method Principle

Residues of clopyralid and picloram are extracted from water matrices by acidifying with 1 N hydrochloric acid (5 mL) followed by a solid-phase extraction (SPE) clean up. The sample is transferred onto a conditioned 0.2 g Waters HLB column at an approximate rate of 2 mL/min. The sample bottle is rinsed with 1 N hydrochloric acid (1 mL) followed by 15:85 acetonitrile/1 N formic acid (5 mL) and the column washed with the rinse before drying under full vacuum for at least 30 minutes. The column is eluted with 14 mL of dichloromethane (DCM). The extract is evaporated to dryness using nitrogen and reconstituted in methanol/0.1% formic acid in water (10:90). The final extract is filtered through a 0.2-µm PTFE syringe filter and then analyzed by liquid chromatography coupled with negative-ion electrospray ionization tandem mass spectrometry (ESI LC-MS/MS).

Test Substance/Analytical Standard

Analytical Standard[a]	TSN Number	Percent Purity	Re-Certification Date	Reference
Clopyralid	TSN301194	99.5%	21 June 2013	FAPC 11-000104
Picloram	TSN029006-0001	99.7%	06 June 2014	FAPC 12-000067

[a]The molecular formulas and structures for these compounds are given in Table 1. The certificates of analysis are given in Appendix 1.

Equipment, Glassware and Materials

Laboratory Equipment

Gilson 'Microman' and Rainin Pos-D pipettes, Anachem Ltd

Vortex mixer, Whirlimixer, Fisher Scientific

Ultrasonic Bath, CamSonix C1275, Camlab

Glassware and Materials

Centrifuge tube, borosilicate glass 16 x 125 mm, Pyrex

HLB column, 6 mL, 200 mg, Part number WAT106202, Waters

0.2 µm PTFE filters, VWR

Chromatographic System

CTC Analytics HTS-Pal Autosampler

Liquid chromatograph, Agilent 1100 Degasser, Binary Pump and Column Oven

Column, Accucore Phenyl-hexyl, 4.6 mm × 50 mm, 2.6 µm, Thermo Scientific

Mass spectrometer, Applied Biosystems API 5000 Triple Quadrupole Mass Spectrometer, electrospray, (ESI, TurboIon Spray)

Software, Applied BioSystems/MDS Sciex Analyst, version 1.6.1

Reagents

Acetonitrile (HPLC grade), formic acid (VWR)

Water (HPLC grade) (Rathburn Chemicals Ltd)

Dichloromethane, methanol (HPLC grade), hydrochloric acid (Fisher Scientific)

Prepared Solutions

Solutions were prepared according to the method as follows:

1 N formic acid: Add 40 mL of concentrated formic acid to a 1 L graduated cylinder containing approximately 500 mL of water and bring to volume with water.

1 N formic acid/acetonitrile (85:15): Place 15 mL of acetonitrile in a 100 mL volumetric flask containing approximately 80 mL of 1 N formic acid, and bring to volume with 1 N formic acid.

0.1% formic acid in water: Place 1000 mL of water in a 1 L bottle and add 1 mL of formic acid.

Methanol/0.1% formic acid (10:90): Place 100 mL of methanol in a reagent bottle and add 900 mL of 0.1% formic acid. Mix well.

1 N hydrochloric acid: Add 83 mL of hydrochloric acid to a 1 L graduated cylinder containing approximately 500 mL of water and bring to volume with water.

Water containing 0.01% formic acid: Place 1000 mL of water in a 1 L bottle and add 0.1 mL of formic acid.

Methanol/acetonitrile (60:40) containing 0.01% formic acid: Place 600 mL of methanol and 400 mL of acetonitrile in a 1 L bottle and add 0.1 mL of formic acid.

EXPERIMENTAL

Instrument Conditions

The instrumental conditions used during the ILV trial were as described in the method with minor adaptations. The instrumental conditions used are given below:

Typical Liquid Chromatography Operating Conditions

Column:	Accucore Phenyl-hexyl, 4.6 × 50 mm, 2.6 µm
Column Temperature:	30 °C
Injection Volume:	50 µL
Flow Rate:	1 mL/min
Mobile Phase A:	HPLC grade water containing 0.01% formic acid
Mobile Phase B:	Methanol/acetonitrile (60:40) containing 0.01% formic acid

Time – minutes	%A	%B
0.00	79	21
2.00	79	21
2.10	5	95
3.50	5	95
3.60	79	21
5.60	79	21

Typical Mass Spectrometry Operating Conditions

Ion Source:	Electrospray, (ESI, TurboIon Spray)
Polarity:	Negative
Collision Gas (CAD):	6
Curtain Gas (CUR):	10
Ion Source Gas 1 (GS1):	50
Ion Source Gas 2 (GS2):	50
IonSpray Voltage (IS):	-4500
Temperature (TEM):	500
Entrance Potential (EP):	-10

Analytes:	Precursor Ion Q1	Product Ion Q3	Dwell Time (msec)	Collision Energy (CE)	Declustering Potential (DP)	Collision Cell Exit Potential (CXP)
Clopyralid (quantification)	190.0	146.0	100	-12	-35	-15
Clopyralid (confirmation)	191.9	147.9	100	-12	-35	-15
Picloram (quantification)	241.0	196.8	100	-14	-35	-15
Picloram (confirmation)	239.0	194.9	100	-27	-35	-15

Scan mass spectra and product ion mass spectra for clopyralid and picloram are shown in Figure 1 through Figure 4.

Typical calibration curves using two ion transitions for the determination of clopyralid and picloram and typical chromatograms are presented in Figure 5 through Figure 8.

Linear regression calculation was performed by the Analyst software, with 1/x weighting, using the concentration in ng/mL for the X-axis, versus the analyte peak area for the Y-axis.

Preparation of Standards

Preparation of Stock Solutions

Stock solutions containing approximately 1000 µg/mL of picloram were prepared by dissolving approximately 50 mg of the reference item in 50 mL of methanol.

Stock solutions containing approximately 1000 µg/mL of clopyralid were prepared by dissolving approximately 50 mg of the reference item in 50 mL of methanol.

Preparation of Fortification Standards

Mixed fortification solutions containing 0.1 and 0.03 µg/mL of picloram and clopyralid were prepared by serial volumetric dilution of stock solutions with methanol.

Preparation of Calibration Standards

Mixed calibration solutions of clopyralid and picloram were prepared by dilution with methanol/0.1% formic acid (10:90) according to the following table:

Use solution with	Volume taken (mL)	Dilute to (mL)	Concentration of each analyte (ng/mL)
1 µg/mL	2.5	50	50
1 µg/mL	2.5	100	25
1 µg/mL	1	100	10
25 ng/mL	4	50	2
10 ng/mL	5	50	1

Preparation of Matrix-Matched Calibration Solutions

For the preparation of matrix-matched standards, 5 extra aliquots of a control extract were taken through the SPE clean up steps, evaporated and reconstituted in 1 mL of each of the calibration standards.

Matrix-matched standard solutions were analyzed together with calibration solutions in solvent by LC-MS/MS. Matrix effects were demonstrated to be insignificant ($\leq$ 10 %) for clopyralid and picloram in all matrices. For details see Table 8 through Table 13.

Sample Origin and Storage

The drinking water specimen was obtained from a 'drinking water' tap at Battelle UK Ltd, Ongar. The ground water specimen was bottled still spring water. The surface water specimen was collected from a pond at Boarded Barns Farm, Ongar, Essex, CM5 0HJ. The drinking water and ground water specimens were stored frozen prior to analysis and the surface water specimen was stored refrigerated prior to analysis. The water specimens were characterized by Agvise, Northwood, ND, 58267 and characterization details are presented in Appendix 4.

Analysis Procedure for the Determination of Clopyralid and Picloram

Extraction Procedure

1. Measure 100 mL of each sample into individual glass bottles equipped with caps.

Note: all steps in the procedure should be carried out in glass containers.

2. For recovery samples, add appropriate aliquots of spiking solution. Refer to table below for example fortification levels.

Sample Description	Spiking Volume	Spiking Solution	Fortification Level
	µL	µg/mL	µg/L
LOD	50	0.03	0.015
LOQ	50	0.1	0.05
10 x LOQ	500	0.1	0.5

3. Sample Purification and Extraction using the following HLB procedure:
    a. Add 5 mL of 1N HCl to samples. Check the pH is below pH 2. Adjust with more HCl if necessary.
    b. Condition 0.2 g Waters HLB columns with 5 mL of MeOH followed by 5 mL of 1N HCl. Pull dry for about 10 seconds.
    c. Transfer the sample solutions onto the HLB columns at a rate of approximately 2 mL/min.
    d. Rinse the sample bottles with 1 mL of 1N HCl. Wash the HLB columns with the rinse.
    e. Rinse the sample bottles with 5 mL of 15:85, ACN/1 N formic acid. Wash the HLB columns with the rinse, then pull dry for at least 30 minutes under full vacuum
    f. Elute the sample from the columns with 14 mL of DCM, collecting the eluate in a glass test tube.

Note: The HLB columns should be profiled in the presence of matrix to determine quantitative analyte recovery with this load/wash/elute pattern.

4. Evaporate the DCM to dryness at ≤40 °C using nitrogen blow down.
5. Reconstitute the samples in 1.0 mL of 10:90, MeOH/0.1% formic acid (5.0 mL for 10 x LOQ samples) with sonication and vortexing. This step is critical in dissolving all residues from the sides of the tube and should be done individually by hand and repeated 2-3 times alternating vortexing and sonication.
6. Filter final extracts through 0.2 µm PTFE syringe filters.
7. Analyze the calibration standards and samples by negative-ion ESI LC-MS/MS, injecting the calibration standards interspersed with the samples throughout the run.

Calculations

Calculations for instrumental analysis were conducted using a validated software application (Applied BioSystems/MDS Sciex Analyst, version 1.6.1) to create a standard curve based on linear regression. The regression functions were used to calculate a best-fit line (from a set of standard concentrations in ng/mL versus peak area response) and to determine concentrations of the analyte found during sample analysis from the calculated best-fit line. For each analytical batch, five calibration standards were injected over the range 1.0 ng/mL to 50 ng/mL. All standards injected and their corresponding peak responses were entered into the program to create the standard curve. Weighting (1/x) was used. With no weighting, the slope of the line (curve) tends to be dominated by the highest point. When weighting of 1/concentration (1/x) is used, the slope more closely approximates the majority of the points used to construct it.

The equation used for the least squares fit is:

$$Y = \text{slope} \times X + \text{intercept}$$

Y = detector response (peak area)

$$X = \frac{Y - \text{intercept}}{\text{slope}} = \text{ng/mL}$$

The standard (calibration) curve generated for each analytical set was used for the quantitation of clopyralid and picloram in the samples from the set. For this study, the correlation coefficient (r) for each calibration curve was equal to or greater than 0.996 (r^2 equal to or greater than 0.992).

For the determination of clopyralid and picloram in terms of µg/L, the following equation was used:

$$\text{Residue (ng/mL} \equiv \text{µg/L)} = \frac{\text{(Residue in Final Volume (ng/mL)} \times \text{Extract Volume (mL)} \times \text{Final Volume (mL)} \times \text{Dilution Factor)}}{\text{(Aliquot Volume (mL)} \times \text{Initial Volume (mL))}}$$

Example: clopyralid recovery of a drinking water sample at 0.05 µg/L (YR12023-1-4).

The concentration determined from the standard curve is = 4.951 ng/mL (as per Analyst 1.6.1)

The residue of clopyralid in the final solution is calculated as follows:

$$\text{Residue µg/L} = \frac{(4.951\ \text{(ng/mL)} \times 1\ \text{(mL)} \times 1\ \text{(mL)} \times 1)}{(1\ \text{(mL)} \times 100\ \text{(mL)})} = 0.04951$$

Procedural recovery data from fortified samples are calculated via the following equation:

Recovery = (Residue (µg/L) / Fortification (µg/L)) × 100 %

Recovery = (0.04951 (µg/L) / 0.05 (µg/L)) × 100 = 99%

Confirmation of Residue Identity

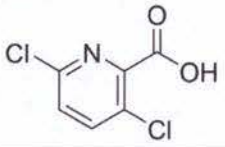
The presence of the analytes are confirmed by comparing the liquid chromatography retention time of the analytes in the calibration standards with those found in the samples, while monitoring two structurally characteristic MS/MS transitions.

clopyralid	*m/z* Q1/Q3 190/146 (quantitation) *m/z* Q1/Q3 192/148 (confirmation)
picloram	*m/z* Q1/Q3 241/197 (quantitation) *m/z* Q1/Q3 239/195 (confirmation)

Statistical Treatment of Data

The mean recoveries for the fortified samples were calculated using the "AVERAGE" function of the Microsoft Excel spreadsheet computer program, which divides the sum of the selected cells by the number of determinations. The standard deviation of the recoveries for a fortification level of one matrix type was calculated using the "STDEV" function of the same spreadsheet program, which sums the squares of the individual deviations from the mean, divides by the number of degrees of freedom, and extracts the square root of the quotient. Percent relative standard deviation, % RSD, was calculated by dividing the standard deviation by the average, and then multiplying by 100. Statistical treatment of data included also the calculation of regression equations and the correlation coefficients (r) for describing the linearity of the calibration curves (Analyst software).

Table 1 Structure of Test / Reference Item

Clopyralid	
Chemical name:	3,6-dichloropicolinic acid
Molecular formula:	$C_6H_3Cl_2NO_2$
Molecular weight:	192.00
Picloram	
Chemical name:	4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid
Molecular formula:	$C_6H_3Cl_3N_2O_2$
Molecular weight:	241.46

Appendix 2 Method Flow Chart

Measure 100 mL of each sample into individual glass bottles equipped with caps.
Note: all steps in the procedure should be carried out in glass containers. For recovery samples, add appropriate aliquots of spiking solution

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Add 5 mL of 1N HCl to samples. Check the pH is below pH 2. Adjust with more HCl if necessary.

↓

Purify samples using the following HLB procedure:

Condition 0.2 g Waters HLB columns with 5 mL of MeOH followed by 5 mL of 1N HCl. Pull dry for about 10 seconds.

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Transfer the sample solutions onto the HLB columns at a rate of approximately 2 mL/min.
Rinse the sample bottles with 1 mL of 1N HCl. Wash the HLB columns with the rinse.
Rinse the sample bottles with 5 mL of 15:85, ACN:1N formic acid.
Wash the HLB columns with the rinse, then pull dry for at least 30 minutes under full vacuum.

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Elute the columns with 14 mL of DCM, collect in a clean glass test tube.

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Evaporate the DCM to dryness at ≤40 °C using nitrogen blow down.

↓

Reconstitute the samples in 1 mL of 10:90, MeOH:0.1% formic acid with sonication and vortexing. This step is critical in dissolving all residues from the sides of the tube and should be done individually by hand and repeating alternating vortexing and sonication 2-3 times.

↓

Filter final extracts through 0.2-µm PTFE syringe filters.

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Analyze the calibration standards and samples by LC-MS/MS injecting the calibration standards interspersed with the samples throughout the run.