INTRODUCTION

Analytical method GRM 07.09, "Determination of Residues of Aminopyralid in Soil by Liquid Chromatography with Tandem Mass Spectrometric Detection" (Appendix A), was developed and validated at Dow AgroSciences LLC. The method was found to be suitable for the determination of residues of aminopyralid in soil over the concentration range of 0.001-0.2 mg/kg. The validated limit of quantitation was 0.001 mg/kg.

An independent laboratory validation of method GRM 07.09 was conducted on one loamy and one clay loam soil type to satisfy the requirements of the Subdivison N (Environmental Fate), Series 164-1; Publication of Addenda for Data Reporting E, K, and N Requirements for Pesticide Assessment Guidelines; Guideline OPPTS 850.7100 "Public Draft"; PR Notices 96-1 and 86-5 and EU Council Directive 91/414/EEC, SANCO/825/00 rev. 7.

The independent laboratory, the Study Director, and the analysts chosen to conduct the ILV were unfamiliar with the method, both in its development and subsequent use in analyzing samples. The independent laboratory used all of its own equipment and supplies, so that there was no common link between Dow AgroSciences and the ILV analysts. Throughout the conduct of the study, any communications between Dow AgroSciences and the Study Director and/or the analyst were logged for inclusion in the report. No one from Dow AgroSciences was allowed to visit the independent laboratory during the ILV trial to observe, offer help, or assist the chemists or technicians. These steps successfully maintained the integrity of the ILV study.

ANALYTICAL

Preparation and Storage of Samples

Two European standard soils (one loamy and one clay loam) were obtained from LUFA Speyer[1] sieved (2 mm). Prior to use in this study, water was added as to establish approximately 40 % of the maximum water holding capacity. Soil characterization information is given in Appendix C.

Preparation of Solutions and Standards

Reagents (obtained from Merck, Fluka, Sigma-Aldrich, Riedel de Haën and Promochem) used were of equivalent specifications as described in Section 6.1 of method GRM 07.09. Solutions were prepared as described in Section 6.3 of method GRM 07.09.

The following analytical test substance/analytical standard was obtained by the sponsor and was utilized during the independent laboratory method validation:

Test Substance/ Analytical Standard	AGR/TSN Number	Percent Purity	Certification Date	Reference
Aminopyralid (4-amino-3,6-dichloro-2-pyridinecarboxylic acid)	TSN102298	99.9%	06-Sept-2006 Re-Certification: 28-Aug-2010	FAPC 063418

The independent laboratory was supplied by the sponsor with the stable isotope to be used as an internal standard, $^{13}C_2{}^{2}H^{15}N$-aminopyralid (4-amino-3,6-dichloropicolinic acid-1-^{15}N-2,6-^{13}C-5-*d*). Standard solutions and calibration standard solutions were prepared as described in Section 7 of method GRM 07.09.

The identity and structures of aminopyralid and related compounds is given in Appendix B.

[1] Landwirtschaftliche Untersuchungs- und Forschungsanstalt Speyer, D-67346 Speyer.

Fortification of Recovery Samples

One ILV trial of the method was run and consisted of the following:

1 reagent blank (containing no matrix or analyte)

2 unfortified control samples

5 control samples fortified at 0.001 mg/kg with aminopyralid (the LOQ of the method)

5 control samples fortified at 0.01 mg/kg with aminopyralid (10 x LOQ).

Fortification solutions were prepared as described in Section 7.1 of the residue analytical method GRM 07.09.

Sample Extraction, Purification and Analysis

The ILV trial was conducted as described in Section 9.3 of method GRM 07.09, with negligible variations due to slightly different laboratory equipment and practices.

As described in Section 11.4 of method GRM 07.09, the solid-phase plate elution profile of aminopyralid was determined. Results confirmed the suitability of the elution procedure described in Section 9.3.20 of method GRM 07.09.

Analytical Instrumentation and Equipment

Prior to initiation of the first ILV trial, the independent laboratory conducted preliminary studies necessary for establishing acceptable performance of the chromatographic instrumentation to be used. These preliminary studies included establishing that adequate HPLC retention times of the analytes and MS/MS detector sensitivity could be achieved. Verification of a lack of aminopyralid contamination in the control sample matrices was not conducted prior to the method trial.

The instrumental conditions used during the ILV trial were conducted as described in Section 8 of method GRM 07.09, with minor adaptations as given below:

Liquid Chromatography Operating Conditions

Instrumentation:	CTC Analytics HTC PAL Autosampler Agilent Model 1100 binary pump Agilent Model 1100 degasser
Column:	Zorbax SB-C8 75 x 4.6 mm, 3.5-µm particle size Securityguard: Phenomenex, C18, 4 x 3 mm,
Column Temperature:	40°C
Injection Volume:	50 µL
Mobile Phase:	A – water with 0.05% formic acid and 5 mM ammonium formate B – methanol with 0.05% formic acid and 5 mM ammonium formate
Flow Rate:	500 µL/min

Gradient:

Time, min	A, %	B, %
0.00	40	60
1.00	40	60
4.00	0	100
9.00	0	100
9.10	40	60
12	40	60

Mass Spectrometry Operating Conditions

Instrumentation:	Applied Biosystems API 3000 LC/MS/MS System Applied Biosystems Analyst 1.4.2 data system
Interface:	TurboIonSpray
Scan Type:	MRM
Resolution:	Q1 – Unit, Q3 – Unit
Nebulizer Gas (NEB)	13
Curtain Gas (CUR):	12
Collision Gas (CAD):	4
Temperature (TEM):	450°C
Polarity:	Positive
IonSpray Voltage (IS):	4500 V
Declustering Potential (DP):	35
Focusing Potential (FP):	180
Entrance Potential (EP):	10

Analytes:	Ion, m/z		Dwell Time, ms	CE/CXP, V
	Q1	Q3		
aminopyralid BE (quantitation)	263.1	134.1	150	57/10
aminopyralid BE (confirmation 1)	263.1	161.1	150	40/12
aminopyralid BE (confirmation 2)	263.1	189	150	25/20
$^{13}C_2{}^2H^{15}N$-aminopyralid BE	269.1	195	150	26/18

Calculations

Linear regression equations were generated for aminopyralid BE calibration standards, separately for the primary quantitation MRM, the confirmatory 1 and confirmatory 2 MRM (Figure 1). Regression calculation was performed by the Analyst software, with 1/x weighting, using the residue concentration ratio (analyte)/(internal standard), in (mg/kg)/(mg/kg), for the X-axis, versus the peak area ratio (analyte peak area)/(internal standard peak area) for the Y-axis.

Calibration standards (see Figure 2 to Figure 3 for examples) with 0.15, 0.5, 1.25, 2.50, 5.0, 12.5, 25.0, 50.0, 100.0 and 125.0 ng/mL of aminopyralid corresponding to 0.03, 0.100, 0.250, 0.500, 1.00, 2.50, 5.00, 10.0, 20.0 and 25.0 ng/mL of derivatized standard (aminopyralid BE), all containing 0.10 mg/kg of internal standard (equivalent to 10 ng/mL) were prepared in methanol/water with 0.05 % formic acid and 5 mM ammonium formate (see GRM 07.09, Section 7.3).

The concentrations of the above calibration standards corresponded to the following residue concentrations in soil: 0.0003, 0.001, 0.0025, 0.0050, 0.010, 0.025, 0.050, 0.100, 0.200 and 0.250 mg/kg (see Section 7.3.1 of method GRM 07.09).

Concentrations of aminopyralid in the final extracts (resulting in mg/kg residue results) were determined by substituting the peak area ratios into the linear regression equation as shown below:

$Y = aX + b$

Y: Ratio: (Analyte peak area / IS peak area)

X: Ratio: (Analyte concentration (c_{End})/ IS concentration)

The IS concentration was always equivalent to 0.10 mg/kg, in calibration solutions as well as in final extracts.

Thus:

$c_{End} = ((Y - b) / a) \times IS\ concentration$

$c_{End} = (((Analyte\ peak\ area / IS\ peak\ area) - b) / a) \times IS\ concentration$

The analyte concentration is thus obtained as residue R in mg/kg.

During method validation, no mass spectral isotopic crossover was observed and therefore no correction of the measured quantitation ratio (as described in Section 10.1 of method GRM 07.09) was performed.

Recoveries (Rec.) were calculated for the fortified specimens as follows:

Rec. = $(R / R_{fortified}) \times 100\ \%$

Example for Calculation

The calculation is exemplified with the clay loam soil specimen PTRL-ID P1465-55.

5 g soil dry mass equivalents were fortified at 0.01 mg/kg (10xLOQ) by dosing 0.5 mL of the 0.10 µg/mL aminopyralid fortification solution.

After clean-up the V_{End} was adjusted to 1.0 mL

The final extract was examined by LC/MS/MS in run file P1465-035 (Figure 7), resulting a aminopyralid BE peak area of 27100 counts (resulting in 0.0085 mg/kg) for the quantitation m/z 263->134. The internal standard peak area in the 269 m/z → 195 m/z MRM ion transition was 887000 counts (equivalent to 10 ng/mL or 0.10 mg/kg).

The Analyst software used the calibration function

Y = 0.364 x X + - 0.000443 (Figure 1, top)

which was established by injecting calibration solutions interspersed with final extracts, whereby:

Y: Ratio: (Analyte peak area / IS peak area)

X: Ratio: (Analyte concentration / IS concentration)

IS concentration was always 0.10 mg/kg, in calibration solutions and in final extracts.

Including the intercept b with - 0.000443, the linear calibration function becomes:

c_{End} = ((Y –b) / a) x IS concentration

= (((Analyte peak area / IS peak area) + 0.000443) / 0.364) x 0.10 mg/kg

= (((27100 counts / 887000 counts) + 0.000443) / 0.364) x 0.10 mg/kg

= 0.0085 mg/kg

Because calibration standard concentrations were expressed in mg/kg the analyte concentration is thus obtained as residue R in mg/kg without needing further calculations.

Recoveries (Rec.) were calculated for the fortified specimens as follows:

Rec. = $(R / R_{fortified}) \times 100\ \%$

= (0.0085 mg/kg / 0.01 mg/kg) x 100 %

= 85 %

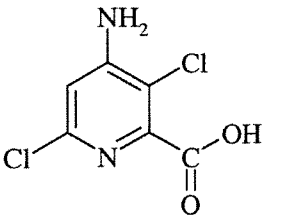
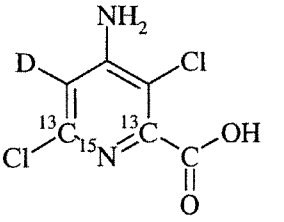
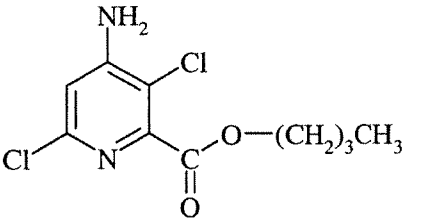
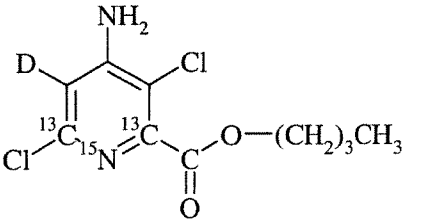
Statistical Treatment of Data

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 mean, and then multiplying by 100.

Confirmatory Evaluation

The presence of aminopyralid determined as aminopyralid BE is confirmed by comparing the liquid chromatography retention times of the analytes in the calibration standards with those found in the samples and by monitoring three characteristic MS/MS transitions.

Appendix B Identity and Structures of Aminopyralid and Related Compounds

Common Name of Compound	Structure and CAS Name
Aminopyralid Molecular Formula: $C_6H_4Cl_2N_2O_2$ Formula Weight 207.02 Nominal Mass: 206 CAS Number: 150114-71-9	 4-amino-3,6-dichloro-2-pyridinecarboxylic acid
$^{13}C_2{}^2H^{15}N$-Aminopyralid Molecular Formula: $^{13}C_2C_4{}^2HH_3Cl_2{}^{15}NNO_2$ Formula Weight 211.00 Nominal Mass: 210 CAS Number: not available	 4-amino-3,6-dichloro-2-pyridinecarboxylic acid-1-^{15}N-2,6-^{13}C-5-*d*
Aminopyralid 1-Butyl Ester Molecular Formula: $C_{10}H_{12}Cl_2N_2O_2$ Formula Weight 263.12 Nominal Mass: 262 CAS Number: not available	 4-amino-3,6-dichloro-2-pyridinecarboxylic acid, 1-butyl ester
$^{13}C_2{}^2H^{15}N$-Aminopyralid 1-Butyl Ester Molecular Formula: $^{13}C_2C_8{}^2HH_{11}Cl_2{}^{15}NNO_2$ Formula Weight 267.11 Nominal Mass: 266 CAS Number: not available	 4-amino-3,6-dichloro-2-pyridinecarboxylic acid-1-^{15}N-2,6-^{13}C-5-*d*, 1-butyl ester