# INTRODUCTION

Analytical method GRM 07.10, "Determination of Residues of Aminopyralid in Drinking Water, Ground Water, and Surface Water 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 water over the concentration range of 0.05-5.00 µg/L. The validated limit of quantitation was 0.05 µg/L.

An independent laboratory validation of method GRM 07.10 was conducted on drinking (tap), surface (pond), and ground (well) water 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

*Drinking (Tap) Water*

Water was collected on 09-Apr-08 in the morning from the PTRL Europe laboratory tap located in Ulm, in Southern Germany. The appearance of the water was clear without any smell. The water was characterized for physical and chemical properties as follows: pH 6.8, total water hardness: 13.2°d (Deutsche Härtegrade, 2.4 mmol/L), dissolved organic carbon (DOC): 0.79 mg/L, turbidity: 0.23 NTU.

*Surface (Pond) Water*

Water was collected on 08-Apr-08 from a pond in Bad Schussenried, located in Southern Germany. The appearance of the water was yellowish. The water was characterized for physical and chemical properties as follows: pH 7.2, total water hardness: 14.2°d (Deutsche Härtegrade, 2.5 mmol/L), dissolved organic carbon (DOC): 3.7 mg/L, turbidity: 105 NTU, silt content: 430 mg/L.

*Ground (Well) Water*

Water was collected on 8-Apr-08 from a spring located in near of Bad Schussenried, in Southern Germany. The appearance of the water was clear. The water was characterized for physical and chemical properties as follow: pH 6.7, total water hardness: 22.4°d (Deutsche Härtegrade, 4 mmol/L), dissolved organic carbon (DOC): 0.89 mg/L, turbidity: 0.60 NTU.

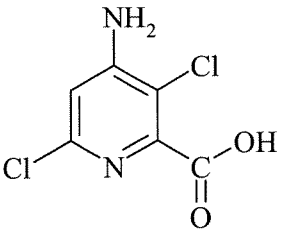
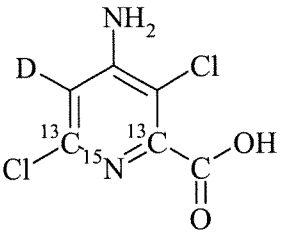
Upon receipt, all the control water matrices were stored refrigerated all times except when removed from the refrigerator for analysis.

## Preparation of Solutions and Standards

Reagents (obtained from Merck, Fluka, Sigma-Aldrich and Promochem) used were of equivalent specifications as described in Section 6.1 of method GRM 07.10. Solutions were prepared as described in Section 6.3 of method GRM 07.10.

The following analytical standards (obtained from the Sponsor) were utilized during the independent laboratory method validation:

**Aminopyralid and Related Compounds**

| Common Name of Compound | Structure and CAS Name |
|---|---|
| Aminopyralid (Test Substance)<br><br>Molecular Formula: $C_6H_4Cl_2N_2O_2$<br>Formula Weight 207.02<br>Nominal Mass: 206<br><br>CAS Number: 150114-71-9 |  <br>4-amino-3,6-dichloro-2-pyridinecarboxylic acid |
| $^{13}C_2{}^2H^{15}N$-Aminopyralid (Internal Standard)<br><br>Molecular Formula: $^{13}C_2C_4{}^2HH_3Cl_2{}^{15}NNO_2$<br>Formula Weight 211.00<br>Nominal Mass: 210<br><br>CAS Number: not available |  <br>4-amino-3,6-dichloro-2-pyridinecarboxylic acid-1-$^{15}$N-2,6-$^{13}$C-5-*d* |

| 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<br>Re-Certification: 28-Aug-2010 | FAPC 063418 |

Standard solutions and calibration standard solutions were prepared as described in Section 7 of method GRM 07.10.

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 0.050 µg/L with aminopyralid (the LOQ of the method)

5 control samples fortified at 0.50 µg/L with aminopyralid (10 x LOQ).

Fortification solutions were prepared as described in Section 7.1 of the residue analytical method GRM 07.10.

Sample Extraction, Purification and Analysis

The ILV trial was conducted as described in Section 9.3 of method GRM 07.10, with negligible variations due to slightly different laboratory equipment and practices.
As described in Section 11.4 of method GRM 07.10, the solid-phase plate elution profile of aminopyralid was determined. Results confirmed the suitability of the elution procedure described in Section 9.3.4 of method GRM 07.10.

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 07.10, with minor adaptations as given below:

Liquid Chromatography Operating Conditions

| | | | |
|---|---|---|---|
| Instrumentation: | CTC Analytics HTC PAL Autosampler<br>Agilent Model 1100 binary pump<br>Agilent Model 1100 degasser | | |
| Column: | Zorbax SB-C8 75 x 4.6 mm, 3.5-µm particle size<br>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<br>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 |
| 12.00 | 40 | 60 |

Mass Spectrometry Operating Conditions

| | |
|---|---|
| Instrumentation: | Applied Biosystems API 3000 LC/MS/MS System<br>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 | 189.0 | 150 | 25/20 |
| aminopyralid BE (confirmation 1) | 263.1 | 161.1 | 150 | 40/12 |
| aminopyralid BE (confirmation 2) | 263.1 | 134.1 | 150 | 57/10 |
| $^{13}C_2{}^2H^{15}N$-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 (µg/L)/(ng/mL), 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 10 ng/mL of internal standard were prepared in methanol/water with 0.05 % formic acid and 5 mM ammonium formate (see GRM 07.10, Section 7.3.).

The concentrations of the above calibration standards corresponded to the following residue concentrations in water: 0.015, 0.05, 0.125, 0.250, 0.500, 1.25, 2.50, 5.0, 10.0 and 12.50 µg/L (see Section 7.3 of method GRM 07.10).

Concentrations of aminopyralid in the final extracts (resulting in µg/L 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 10 ng/mL, in calibration solutions as well as in final extracts.

Thus:

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

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

The analyte concentration is thus obtained as residue R in µg/L.

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 07.10) 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 surface water specimen PTRL-ID P1464-62.

10 mL of surface water were fortified at 0.5 µg/L (10xLOQ) by dosing 0.05 mL of the 0.10 µg/mL aminopyralid fortification solution.

After clean-up the $V_{End}$ was adjusted to 5.0 mL

The final extract was examined by LC/MS/MS in run file P1464-088 (Figure 7), resulting in a aminopyralid BE peak area of 129000 counts (equivalent to 0.48 µg/L) for the quantitation m/z 263->189. The internal standard peak area in the 269 m/z → 195 m/z MRM ion transition was 769000 counts.

The Analyst software used the calibration function

Y = 3.5 x X + - 0.000897 (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)
with IS concentration always 10 ng/mL or µg/L.

Including the intercept b with - 0.000897, the linear calibration function becomes:

$c_{End}$ = ((Y –b) / a) x IS concentration
= (((Analyte peak area / IS peak area) + 0.000897) / 3.5) x 10 µg/L
= (((129000 counts / 769000 counts) + 0.000897) / 3.5) x 10 µg/L
= 0.48 µg/L

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

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

Rec. = ($R / R_{fortified}$) x 100 %
= (0.48 µg/L / 0.50 µg/L) x 100 %
= 96 %

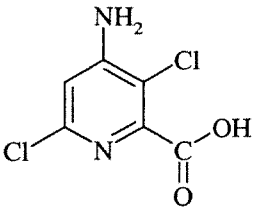
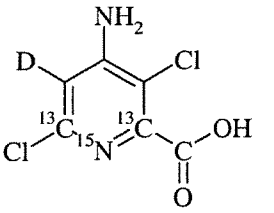
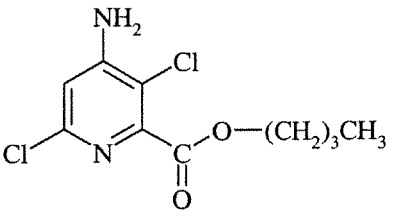
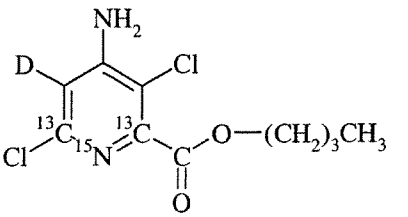
Statistical Treatment of Data

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.

Table 1. Identity and Structures of Aminopyralid and Related Compounds

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