Independent Laboratory Validation of Dow AgroSciences Method GRM 02.34—
Determination of Residues of Aminopyralid in Soil by Liquid Chromatography
with Tandem Mass Spectrometry

INTRODUCTION

An independent laboratory validation (ILV) study was conducted following both a draft and the final version of Dow AgroSciences LLC residue analytical method GRM 02.34, "Determination of Residues of Aminopyralid in Soil by Liquid Chromatography with Tandem Mass Spectrometry Detection", dated November 26, 2003 (Appendix A) and January 6, 2004 (Appendix B), respectively. For the purpose of conducting the independent laboratory validation, the draft method is essentially the same as the final version with an effective date of January 6, 2004. The ILV study was conducted to fulfill the requirements described in U.S. EPA guidelines

(References 1, 2, 3 and 4). The control sample (a sandy loam soil) selected for evaluation was one of the control soils remaining from the U.S. field dissipation study. This soil sample was selected as it is a soil representative of the growing area where aminopyralid would be applied.

Method GRM 02.34 was developed and initially validated at Dow AgroSciences. The independent laboratory, the Study Director, and the analysts chosen to conduct the ILV were unfamiliar with the method, both in its development and in its subsequent use in analyzing field samples. The independent lab used its own equipment and supplies, so that there was no common link in equipment between Dow AgroSciences and the Study Director and/or the analysts. Throughout the conduct of the study, any communications between Dow AgroSciences and the Study Director and/or the analysts were logged for inclusion in the report. No one from Dow AgroSciences visited Morse Laboratories, Inc., during the ILV trial to observe, offer help, or to assist with the method evaluation. These steps successfully maintained the integrity of the ILV study.

ANALYTICAL

Sample Numbering, Preparation, and Storage

Dow AgroSciences shipped, frozen and prepared, one control soil sample uniquely identified as 37585301. It was stored frozen (approximately -20° ± 5° C) in a temperature-monitored freezer following receipt. The sample was stored frozen (approximately -20° ± 5° C) except when removed to prepare sample aliquots for analysis.

Preparation of Solutions and Standards

All reagent solutions were prepared as described in Subsection 6.3. of method GRM 02.34 with the following exceptions:

Step 6.3.1. acetonitrile/1N hydrochloric acid (90:10) was prepared as follows:

Note: Only 1000 mL of reagent was prepared rather than 2000 mL as specified in the method.

To a 1-L volumetric flask, ~500 mL of acetonitrile, followed by 100 mL of 1N HCl, were added. The contents were gently mixed, then brought to volume with acetonitrile. The mixture was allowed to cool prior to use.

The following analytical reference standards/test substances were utilized during the independent laboratory method validation:

Standards	AGR/TSN No.	Percent Purity	Certification Date	Reference
aminopyralid (XDE-750)	TSN102298	99.9	04-Oct-2002	FA&PC 023235
aminopyralid-1-^{15}N-2,6-^{13}C[a]	NA	NA	NA	E-1145-19

[a]Stable isotope (internal standard)

Standard solutions were prepared as described in Section 7 of method GRM 02.31 with the following exceptions:

1) Step 7.1.1. The stock solution (1000 µg/mL) of aminopyralid analytical standard was prepared under Dow AgroSciences Study No. 030039.

2) Step 7.1.2. The 100 µg/mL-aminopyralid spiking solution was prepared under Dow AgroSciences Study No. 030039.

3) Step 7.2.1. The stock solution (76 µg/mL) of aminopyralid stable isotope analytical standard was prepared under Dow AgroSciences Study No. 030039.

Note: The concentration prepared, which was less than the 100 µg/mL specified in the method, was due to the smaller amount of analytical standard received from the Sponsor.

4) Step 7.2.2. In order to prepare a 1.0 µg/mL-internal standard (stable isotope) solution in acetonitrile:pyridine:butanol (22:2:1), 6.579 mL of the stock solution (76 µg/mL) was diluted to a final volume of 500 mL.

5) Step 7.2.3. In order to prepare a 1.0 µg/mL-internal standard (stable isotope) solution, in acetonitrile, 1.320 mL of the stock solution (76 µg/mL) was diluted to final volume of 100 mL.

Fortification of Recovery Samples

The ILV trial was conducted using one control soil. The sample set composition was as follows:

One reagent blank
Two unfortified control samples
Five control samples fortified with aminopyralid at 0.0015 µg/g (LOQ)
Five control samples fortified with aminopyralid at 0.015 µg/g (10 × LOQ)

Sample Extraction and Analysis

Residues of aminopyralid were extracted from the soil by shaking with an acetonitrile/1N hydrochloric acid solution (90:10). The sample was centrifuged, and the extract was decanted into a graduated mixing cylinder. A second extraction was performed by adding extraction solution to the soil and shaking the sample on a reciprocating shaker for 30 minutes. The sample was centrifuged and the second extract was combined with the original extract. An aliquot of the extract was evaporated to dryness and reconstituted in 1N hydrochloric acid. An aliquot of the concentrated extract was purified using a polymeric 96-well solid phase extraction (SPE) plate. The SPE plate was washed with a water/methanol solution (95:5) and eluted with acetonitrile. A stable-isotope labeled internal standard ($^{13}C_2{}^{15}N$-aminopyralid) was added to the eluate. The eluate was evaporated to dryness, and the residues were reconstituted in an

acetonitrile/pyridine/butanol solution (22:2:1). The residue was derivatized with butyl chloroformate. After derivatization, the mixture was brought to a final volume of 1.0 mL with methanol/water/acetic acid (50:50:0.1) mobile phase. The purified extract was then analyzed by high performance liquid chromatography with positive-ion electrospray (ESI) tandem mass spectrometry (LC/MS/MS).

All trials were conducted exactly as described in method GRM 02.34 with the following exceptions:

(1) Step 9.3.16. An N-Evap evaporator was substituted for a TurboVap® LV evaporator.

(2) Step 9.3.19.e. Eluates collected from this step were quantitatively transferred from the 96-well collection plate to 13 x 100 mm test tubes and evaporated by use of N-Evap evaporation rather than evaporation directly in the collection plates using the 96-well evaporator. This is considered an equivalent technique.

(3) Step 9.3.20. Evaporated the sample eluate in 13 × 100 mm test tubes using an N-Evap evaporator rather than a 96-well evaporator.

(4) Step 9.3.21. Pipetted 200 µL of acetonitrile/pyridine/butanol (22:2:1) solution into separate 13 × 100 mm test tubes rather than into individual wells of a 96-well collection plate.

(5) Step 9.3.22. Pipetted 200 µL of each calibration standard into separate 13 × 100 mm test tubes rather than into individual wells of a 96-well collection plate.

(6) Step 9.3.23. Pipetted 200 µL of the 0.125-µg/mL aminopyralid cross over standard into a separate 13 × 100 mm test tubes rather than into individual wells of a 96-well collection plate.

(7) Step 9.3.24. Pipetted 200 µL of the 0.125-µg/mL aminopyralid stable isotope standard into separate 13 × 100 mm test tubes rather than into individual wells of a 96-well collection plate.

(8) Step 9.3.25. Derivatize samples, calibration standards, and crossover standards by pipetting 10µL of butyl chloroformate into each test tube instead of the individual wells of a 96-well collection plate

(9) Step 9.3.27. The mobile phase was added to each test tube instead of the individual wells of a 96-well collection plate.

(10) Step 9.3.28 13 × 100 mm test tubes were vortexed instead of the 96-well collection plate.

Analytical Instrumentation

The following instruments were utilized and are considered equivalent to those described in Section 8. of method GRM 02.34:

Instrumentation:

Liquid Chromatograph/Tandem Mass Spectrometer

Mass Spectrometer – PE Sciex API 2000 (MSD-29-02)

Liquid Chromatograph – Two Shimadzu LC10ADvp pumps (PS-20-02, PS-21-02)

Autosampler – Perkin Elmer Series 200 (AS-28-02)

Solvent Degasser – Shimadzu DGU-14A (VB-05-02)

System Controller – Shimadzu SCL10Avp (CN-15-02)

Mass Spectrometer Data System – PE Sciex Analyst 1.1 data system

Computer – Dell Optiplex, GX400 (CT-49-02)

Printer - Laser jet HP4050 printer (CP-37-00)

Operating Parameters:

Liquid Chromatography

Column:	Diazem 3000, C18, 4.6 mm × 100 mm, 3 µm Morse Labs column #511
Column Temperature:	35°C
Injection Volume:	100 µL
Run Time:	9 minutes

Mobile Phase:	A – water with 0.1% acetic acid B – methanol with 0.1% acetic acid
Flow Rate:	900 µL/min, flow diverted to source between 3.0 minutes and 4.5 minutes

Gradient:

Time, min	A, %	B, %
0.0	50	50
5.0-6.0	100	0
6.1-9.0	50	50

Mass Spectrometry (MS/MS)

API 2000:

Interface:	TIS (TurboIonSpray)
Scan Type:	MRM
Resolution:	Q1 – unit, Q3 – low
Curtain Gas (CUR):	40
Collision Gas (CAD):	12
Temperature (TEM):	425 °C
Ion Source Gas 1 (GS1):	45
Ion Source Gas 2 (GS2):	75
Period 1	
Time:	6 minutes
Polarity:	Positive
Ion Spray Voltage	5000

Compound	Ion, *m/z*		Time, *ms*	CE, *v*
	Q1	Q3		
Aminopyralid butyl ester	263.2	134.1	150	49
Aminopyralid stable isotope butyl ester	268.2	139.1	150	49

Analytical Equipment and Materials

Equipment and materials were utilized in the conduct of the independent validation as described in method GRM 02.34 with the following exceptions (which are considered to be equivalent substitutions):

Equipment:

(1) Step 4.1.3. An IEC Clinical centrifuge was substituted for a bench top Model Centra-GP8 centrifuge.

(2) Step 4.1.4. A Model 112 N-Evap evaporator was substituted for a TurboVap evaporator.

(3) Step 4.1.5. A Model 112 N-Evap evaporator and the use of 13 × 100 mm test tubes was substituted for a SPE-Dry-96 evaporator.

(4) Step 4.1.8. A Finnpipette 5-40 µL adjustable pipettor was substituted for an Eppendorf 5-100 µL adjustable pipettor.

(5) Step 4.1.9. A Finnpipette 40-200 µL adjustable pipettor was substituted for an Eppendorf 20-300 µL adjustable pipettor.

(6) Step 4.1.10. A Finnpipette 200-1000 µL adjustable pipettor was substituted for an Eppendorf 50-1000 µL adjustable pipettor.

(7) Step 4.1.13. A Branson Model 2210 ultrasonic cleaner was substituted for a Branson Model 1200 ultrasonic cleaner.

Reagents:

Section 6.1: The following substitutions (which are considered to be equivalent to what was used in the method) were made regarding reagents:

1. Burdick and Jackson HPLC grade acetonitrile was substituted for Mallinckrodt Baker brand

2. EM Science OmniSolv methanol and Burdick and Jackson, B & J Brand® high purity methanol was substituted for Mallinckrodt HPLC grade

3. Burdick and Jackson HPLC grade water and Fisher Scientific HPLC grade water was substituted for EM Science OmniSolv brand

Additional equipment used:

Analytical Balance: Mettler AB104 (B-20-97)

Top-Loading Balance: Mettler PE 1600 (B-02-86)

Centrifuges: IEC Clinical (C-05-89, C-07-95)

Collection plate: 96-well, 2 mL, Phenomenex, Inc.

Pipettor: Finnpipette, adjustable, 5-40 µL, Fisher Scientific (AP-13-94)

Pipettor: Finnpipette, adjustable, 40-200 µL, Fisher Scientific (AP-05-89)

Pipettor: Finnpipette, adjustable, 200-1000 µL, Fisher Scientific (AP-30-96)

Vacuum pump: Welch Gem 1.0 vacuum pump (P-33-97)

Vacuum manifold: 96-well plate acrylic manifold, Phenomenex, Inc. (MC-46-03)

Vortex mixer: Scientific Industries Model G-560 (M-09-86)

Shaker: Platform, Eberbach Model 6000 (SH-08-97, SH-02-88)

Evaporator: N-Evap, Model 112, Organomation Associates, Inc. (SB-07-04)

Culture tubes: 13 × 100 mm and 16 × 100 mm, VWR Scientific

Computer: Dell Optiplex, GX400 (CT-49-02)

Microsoft Excel '97 for Windows, Microsoft Corporation

Vials: 40-mL with PTFE-lined screw caps (Supelco, Cat no. 27181)

Ultrasonic cleaner: Branson Model 2210 (S-04-93)

Calculations

The percent recovery of aminopyralid from soil was calculated as described in Section 10 of method GRM 02.34. Calibration standards for aminopyralid (0.0001 µg/mL, 0.0005 µg/mL, 0.001 µg/mL, 0.005 µg/mL, 0.01 µg/mL, 0.02 µg/mL, 0.035 µg/mL, each containing 0.025 µg/mL of $^{13}C_2{}^{15}N$-aminopyralid stable isotope internal standard) were analyzed with the each sample set. Also, a 0.025 µg/mL aminopyralid stable isotope crossover standard (containing 0.025 µg/mL of $^{13}C_2{}^{15}N$-aminopyralid stable isotope internal standard only) was included in each sample set for isotopic crossover determination. The calculation process involved two steps:

1) determination of isotopic crossover, and 2) calculation of residue found after correction for isotopic crossover.

1. Determination of Isotopic Crossover

In this assay, the analyte and its stable isotope internal standard were quantified using MS/MS transitions characteristic of each compound. When using stable-isotope labeled internal standards, there is a possibility that isotopic contributions (crossovers) will occur between the transition ions used for quantitation of the unlabeled and labeled compounds. As discussed in the method, the only isotopic overlap of consequence is the crossover of the $^{13}C_2{}^{15}N$-aminopyralid stable isotope internal standard to aminopyralid (ISTD→analyte). A 0.025 µg/mL aminopyralid stable isotope crossover standard was analyzed to determine a "crossover factor", which is an internal standard quantitation ratio with respect to isotopic contribution of the $^{13}C_2{}^{15}N$-aminopyralid internal standard to the aminopyralid. It was subsequently used to correct quantitation ratios used to construct the analyte standard curve and determine sample residues. The transitions measured were: *m/z* 263→134 for aminopyralid and *m/z* 268→139 for $^{13}C_2{}^{15}N$-aminopyralid. The crossover factor was calculated as follows using the data derived from the stable isotope crossover standard:

$$\text{Crossover Factor (ISTD} \rightarrow \text{analyte)} = \frac{\text{peak area at } m/z\ 263 \rightarrow 134}{\text{peak area at } m/z\ 268 \rightarrow 139}$$

Example Calculation of Crossover Factor:

The crossover factor used for analytical set #2, soil, was calculated as follows:

Stable isotope crossover standard (ISTD→analyte):
peak area *m/z* 263→134 = 301
peak area *m/z* 268→139 = 67100

$$\text{Crossover Factor (ISTD} \rightarrow \text{analyte)} = \frac{301}{67100} = 0.004486$$

The resulting crossover factor was used to correct the quantitation ratios discussed in the next section.

2. Calculation of Residue Found

A validated software application (GraphPad Prism®, version 3.03) was used to generate a standard curve for aminopyralid from a set of standard concentrations (in µg/mL) versus their respective quantitation ratios. A quantitation ratio for each standard was determined by dividing the peak area for aminopyralid transition (m/z 263→134) by the peak area for the stable isotope internal standard transition (m/z 268→139).

$$\text{Quantitation ratio (uncorrected)} = \frac{\text{peak area of aminopyralid (m/z 263} \rightarrow \text{134)}}{\text{peak area of stable isotope internal standard (m/z 268} \rightarrow \text{139)}}$$

For each standard concentration, the uncorrected quantitation ratio was corrected for the for the isotopic contribution of the $^{13}C_2{}^{15}N$-aminopyralid internal standard to the aminopyralid as follows:

$$\text{Quantitation Ratio (corrected)} = \text{Quantitation Ratio (uncorrected)} - \text{Crossover Factor}$$

A quadratic non-linear regression equation was used to determine concentrations of the analyte found in the sample. It was the most appropriate equation for use in this study that best defined the relationship between the analyte concentration and the quantitation ratios generated, over the range of fortification levels tested, when evaluated using the detector specific to the study. The equation is:

$$y = a + bx + cx^2$$

where:

y	=	corrected quantitation ratio
x	=	µg/mL found for peak of interest
a. b, c	=	factors derived from calculation of standard curve

Note: A standard curve was generated by plotting the standard concentration (in µg/mL) on the x-axis and the respective corrected quantitation ratio on the y-axis.

Using the standard curve generated to determine µg/mL of aminopyralid found, the concentration (in µg/g) of residue in the sample was determined using the following equation:

$$\mu g/g\,(gross) = \mu g/mL \times \left(\frac{(Extraction\ vol.) \times (Final\ vol.)}{(Aliquot\ x\ Aliquot\ factor) \times (Sample\ wt.)} \right) \times Dilution$$

The above equation is equivalent to the following:

$$\mu g/g\,(gross) = \mu g/mL \times \frac{ext.\,solv.\,(mL)}{samp.\,wt.\,(g)} \times \frac{final\ vol.\,(mL)}{aliq.\,1\,(mL)} \times \frac{pre\text{-}SPE\ vol.\,(mL)}{aliq.\,2\,(mL)} \times dil.\,factor$$

where:

µg/mL	=	µg/mL analyte found from standard curve
ext. solv. (mL)	=	extraction solvent volume (40 mL)
samp. wt. (g)	=	gram weight of sample extracted (5.0 g)
aliq. 1 (mL)	=	volume of sample extract taken through procedure (6.0 mL)

pre-SPE vol. (mL)	=	final volume that aliquot 1 is brought to (via concentration and reconstitution) prior to SPE (3.0 mL)
aliq. 2 (mL)	=	volume of pre-SPE reconstituted extract processed through SPE (1.5 mL)
final vol. (mL)	=	final volume submitted to HPLC analysis (1.0 mL)
dil. factor	=	dilution of sample extract required to produce an analyte response bracketed by standards

3. Calculation of Percent Recovery

The following equations were used:

$$\text{net } \mu g/g \text{ in fortified control} = (\mu g/g \text{ fort. cont.}) - \left[\frac{(\mu g/g \text{ cont. } 1) + (\mu g/g \text{ cont. } 2)}{2}\right]$$

$$\% \text{ Recovery} = \frac{\text{net } \mu g/g \text{ in fort. cont.}}{\text{fortification level } (\mu g/g)} \times 100$$

Example Calculations

1. Crossover factor, Soil, Set 2 (Figure 8):

Stable isotope crossover standard (ISTD→analyte):
peak area *m/z* 263→134 = 301
peak area *m/z* 268→139 = 67100

$$\text{Crossover Factor (ISTD} \rightarrow \text{analyte)} = \frac{301}{67100} = 0.004486$$

2. Soil, 3758301, Control 3, Set 2, dil. factor = 1 (Figure 10):

Peak area aminopyralid (*m/z* 263→134): 309
Peak response, internal standard (*m/z* 217): 47200

$$\text{Quantitation ratio (uncorrected)} = \frac{309}{47200} = 0.006547$$

$$\text{Quantitation ratio (corrected)} = 0.006547 - 0.004486 = 0.002061$$

0.002061 from standard curve = 0.000001355145 µg/mL

$$\mu g/g\ (\text{gross}) = 0.000001355145\ \mu g/mL \times \frac{40\ mL}{5.0\ g} \times \frac{1.0\ mL}{6.0\ mL} \times \frac{3.0\ mL}{1.5\ mL} \times 1$$

$$= 0.0000036137$$

Reported µg/g (gross) = 0.00000361

3. Soil, 3758301, Fortified Control 13 @ 0.0015 µg/g, Set 2, dil. factor = 1 (Figure 11):

Peak area aminopyralid (*m/z* 263→134): 2350
Peak response, internal standard (*m/z* 217): 48000

$$\text{Quantitation ratio (uncorrected)} = \frac{2350}{48000} = 0.048958$$

$$\text{Quantitation ratio (corrected)} = 0.048958 - 0.004486 = 0.044472$$

0.044472 from standard curve = 0.0004590961 µg/mL

$$\mu g/g \text{ (gross)} = 0.0004590961\ \mu g/mL \times \frac{40\ mL}{5.0\ g} \times \frac{1.0\ mL}{6.0\ mL} \times \frac{3.0\ mL}{1.5\ mL} \times 1$$

$$= 0.0012242563$$

$$\text{Reported } \mu g/g \text{ (gross)} = 0.00122$$

$$\text{net } \mu g/g \text{ in fortified control} = 0.00122\ \mu g/g - \left[\frac{(0.0000036137\ \mu g/g) + (0.000\ \mu g/L)}{2}\right]$$

$$\text{net } \mu g/g = 0.00122$$

$$\%\ \text{Recovery} = \frac{0.00122\ \mu g/L}{0.0015\ \mu g/L} \times 100$$

$$\text{Recovery} = 81\%$$

Statistical Treatment of Data

Statistical methods used were limited to calculation of the means, standard deviations and relative standard deviations. A validated software program, Microsoft Excel '97, was employed to develop all statistical data.

Problems Encountered and Changes or Modifications Made

The following problem was encountered during the course of the study:

Initial extraction of the samples (Steps 9.3.5. and 9.3.10.) needed to be conducted in a more vigorous manner than that specified in the method by setting the platform shaker at the maximum speed the extraction vessels could tolerate without creating a hazard (i.e., ≥200 excursions/minute rather than just approximately 180 excursions/minute). **Approximately** 180 excursions/minute can be interpreted to include a lower excursion rate (as low as 160 excursions/minute using the ±10% interpretation for approximate) that may not provide for sufficient analyte extraction. This becomes an issue when

equipment is used that may approach, but just not quite reach, the targeted rate (i.e., only achieving 160-170 excursions/minute). Thorough extraction is crucial for acceptable recovery of this analyte from the soil matrix. Morse Labs found that a minimum excursion rate of ≥200 excursions/minute was required to achieve a thorough extraction.

No other problems were encountered nor were changes/modifications made to the method during the course of the study, with the exception of the substitution of some equipment as addressed in the "Analytical Equipment and Materials" section.

Critical Steps

The following steps are considered critical, and must be conducted thoroughly, for a successful analysis.

(1) Steps 9.3.5. and 9.3.10. Particular attention needs to be paid to the extraction steps. For thorough extraction of aminopyralid from soil samples, these steps must be conducted in a vigorous manner (i.e., platform shaker speed set at ≥200 excursions/minute).

Other than ensuring that the initial extraction is conducted in a vigorous manner, no other steps in the method were determined to have to be followed so specifically (or critically) that they required special care and/or specific instructions in order to avoid posing the risk of method failure.