1.0 PURPOSE

The purpose of this study was to validate a method associated with the analysis of Pethoxamid and MET-42 in soil using methodology as described in Method No. M-80127-000, Analytical Method for the Determination of Pethoxamid and MET-42 in Soil via LC/MS/MS (Appendix 2). The study followed the extraction procedure described in the method and used reverse-phase high-pressure liquid chromatography with triple-quad mass spectrometry (LC/MS/MS) detection.

2.0 SUMMARY

Method No. M-80127-000 (Appendix 2) was evaluated for the analysis of Pethoxamid and MET-42 in soil. This evaluation was designed to meet requirements for independent laboratory validations (ILV) as described in U.S. EPA Ecological Effects Test Guidelines, OCSPP 850.6100 (1).

A total of 24 matrix samples were analyzed as part of a method trial, consisting of two unfortified control samples and ten control samples fortified with a mixed standard solution of Pethoxamid and MET-42 for each of two soil types. The fortification levels included ten samples fortified with Pethoxamid and MET-42 at the target limit of quantitation (LOQ) of 10 ppb and ten samples fortified at 100 ppb. Two reagent blanks and four spiked soil matrix samples were also prepared (two soil matrix spikes fortified at the LOQ and two fortified at 10×LOQ.)

The method was successfully validated during the first method trial.

3.0 EXPERIMENTAL DETAILS

3.1 Test Substance

The test substances for this study were Pethoxamid and MET-42.

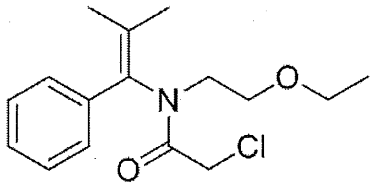
3.2 Analytical Reference Substance

The test substances mentioned in the previous section also served as the analytical reference substances. Information regarding the test and reference substances is summarized below. Certificates of Analysis (COA) are located in Appendix 4.

Pethoxamid:

Chemical Name:	Acetamide, 2-chloro-N-(2-ethoxyethyl)-N-(2-methyl-1-phenyl-1-propen-1-yl)-
CAS Number:	106700-29-2
Empirical Formula:	$C_{16}H_{22}ClNO_2$

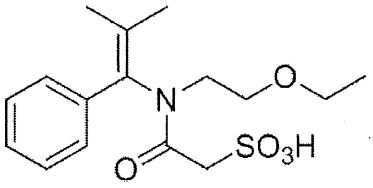
Molecular Weight:	295.80 g/mol
Batch No.:	P1351-BKA-89
GLP Purity:	99.8% w/w
Expiration Date:	12 November 2014
Storage Conditions:	−20°C
Chemical Structure:	



Pethoxamid

MET-42:

Chemical Name:	Ethanesulfonic acid, 2-[(2-ethoxyethyl)(2-methyl-1-phenyl-1-propen-1-yl) amino]-2-oxo-, sodium salt
CAS Number:	1329805-71-1
Empirical Formula:	$C_{16}H_{22}NNaO_5S$
Molecular Weight:	363.40 g/mol
Batch No.:	P1925-cWa-03
GLP Purity:	90.3% w/w
Expiration Date:	24 October 2014
Storage Conditions:	Ambient Desiccator
Chemical Structure:	



MET42

A 1.00 mg/mL purity-corrected stock solution was prepared on 21 March 2014, by transferring 10.040 mg (uncorrected for purity) of Pethoxamid to a 10-mL volumetric flask and bringing to volume with acetone. Concurrently, a 1.00 mg/mL purity corrected stock solution of MET-42 was prepared by

transferring 11.093 mg (uncorrected for purity) to a 10-mL volumetric flask and bringing to volume with acetone. From dilutions of these stock solutions an Intermediate Mixed Standard solution was prepared with 0.1% Formic Acid in 30:70 acetonitrile/water (hereafter referred to as Injection Solvent). Calibration Standards were prepared from dilutions of the Intermediate Standard solution in Injection Solvent. Fortification solutions were prepared from dilutions of the Intermediate Standard solution in Injection Solvent.

All Standard solutions were prepared using Class A volumetric glassware and direct displacement micro-pipettes. Solutions were stored in a refrigerator when not in use. In order to assess the viability of solutions stored under refrigerated conditions, subsequent calibration standard solutions were prepared as described above from fresh standard weighings.

3.3 Method Summary

Control soil samples were weighed into 50-mL calibrated polypropylene (PPE) tubes at 5 ± 0.05 g per sample. The samples were fortified using the Pethoxamid and MET-42 spiking solutions in Injection Solvent at concentrations of 10 and 100 ppb. Steel balls were added to each sample. A graduated cylinder was used to add 15 mL of acetonitrile to each sample. The samples were shaken for 10 minutes at high speed on a reciprocating platform shaker then centrifuged at ≈3000 rpm for ≈5 minutes to form a solid pellet. Sample supernatants were decanted into clean 50-mL PPE centrifuge tubes and the pellet re-suspended in 15 mL of Extraction Solvent (0.1% Formic Acid in 1:1 acetonitrile/water) measured via graduated cylinder. Soil samples were returned to the platform shaker and shaken at high speed for 10 minutes. The centrifugation step was repeated and the supernatants combined with the extracts from the first extraction. The pellet was again re-suspended in an additional 15 mL of Extraction Solvent and the 10 min shake repeated for a total of three extractions. Following the centrifugation step, the supernatant was combined with the first two extracts. The extracts were adjusted to exactly 50 mL with 0.1% Formic Acid (aq). A 1-mL aliquot was removed from each sample and placed in a clean 15-mL graduated PPE tube. The aliquot was then diluted ten-fold by adjusting the final volume to 10-mL with Injection Solution. Extracts were then analyzed by LC/MS/MS.

3.4 Sample Receipt and Storage

ABC Laboratories received approximately 0.50 g each of Pethoxamid and MET-42 from Cheminova A/S, Lemvig, Denmark on 28 May 2013. The Pethoxamid test substance was stored at –20°C and the MET-42 test substance was stored in a desiccator at room temperature.

Soils obtained from Jefferson County, Iowa, and from Willacy County, Texas, were used as the untreated Controls.

3.5 Instrument Conditions

LC/MS/MS Analysis:

Analysis of samples was accomplished using a PESciex 5000 LC/MS/MS System. The instrument parameters were as follows:

Instrument: Applied Biosystems/Sciex API 5000 MS/MS with Waters Acquity System and Autosampler
Column: 50 × 3.0 mm, Synergi Polar-RP with 2.5-µm packing
Column Temp.: 40°C
Injection volume: 10 µL
Autosampler Temp.: 10°C
Mobile Phase A: 0.1% Formic Acid in Water
Mobile Phase B: 0.1% Formic Acid in Methanol
Flow Conditions: No Split

Time (min)	%A	%B	Flow Rate (mL/min)
0	40	60	0.5
2.5	15	85	0.5
4.0	15	85	0.5
4.1	40	60	0.5
6.0	40	60	0.5

Interface: Turbospray
Mode: MRM
Resolution: Unit
TIS Source: Negative (MET-42) & Positive (Pethoxamid)

Analyte	Q1 (m/z)	Q3 (m/z)	Dwell (msec)	CUR (psi)	GS1 (psi)	GS2 (psi)	Temp (°C)	IHE	IS (V)	CAD (psi)	DP (V)	EP (V)	CE (V)	CXP (V)
Pethoxamid	296.2	131.1	300	40	50	50	500	On	3500	8	20	10	29	15
	296.2	250.2											18	15
MET-42	340.0	120.7	300	40	50	50	500	On	−3500	8	−50	−10	−33	−15
	340.0	79.9											−60	−15

3.6 Assignment of Sample Identification

All samples were assigned a unique sample identification number beginning with the five-digit ABC study number (80128) followed by a number that was assigned consecutively as samples were prepared, beginning with the sample number -001. For example, the first sample was assigned the unique identification of 80128-001, the second 80128-002, and so on.

3.7 Calculations

Concentrations of the test substances, Pethoxamid and MET-42, were determined using the External Standard Analysis Function of Analyst software. The

concentrations of test substance in the samples prepared for LC/MS/MS analysis were determined directly from the Calibration Standard curve as shown in the equations below.

For MET-42-1 (ion transition 340.0 → 120.7), the calibration curve was fit linear weighted 1/x such that:

$y = mx + b$

where:

y = peak area in cps

m = the slope of the line from the calibration curve

x = concentration of injected sample in ng/mL

b = the Y-axis intercept of the calibration curve

Example:

For the LC/MS/MS analysis of the MET-42 LOQ fortification of IA control soil, sample 80128-008, the equation for the standard curve of the 340.0/120.7 dalton (Da) ion transition was:

$y = 104376\ x + 1109.02$

The peak area (y) for the injection was 10559 cps Substituting the peak area (y) in the following equation and solving for x gave the concentration of MET-42-1

$x = (10559 - 1109.02) / 104367$

A concentration value (x) of 0.0905 ng/mL was calculated by the Analyst software. The concentration value (x) in ng/mL was then multiplied by the analysis volume (10 mL), the aliquot factor (50 mL extraction vol./1 mL aliquot vol.) and divided by the sample mass (5 g) to result in a final concentration of 9.05 ng/g = 9.05 ppb. That is,

$x\ ppb = (0.0905\ ng/mL) \times 10\ mL \times 50\ mL/1\ mL \div 5\ g = 9.05\ ng/g = 9.05\ ppb$

The percent recovery was calculated by dividing the residue found by the actual fortification level:

$$\% \text{ Recovery} = \frac{9.05 \text{ ppb}}{10 \text{ ppb}} = 91\%$$

For the ion transition 296.2/131.1 Da of Pethoxamid (PETH-1), the calibration curve response was quadratic, therefore:

$y = ax^2 + bx + c$

where:

y = peak area in cps

a and b = the coefficients of the first two terms of the calibration curve's quadratic expression.

x = concentration of injected sample in ng/mL

c = the y-axis intercept of the calibration curve

Example:

For the LC/MS/MS analysis of the Pethoxamid LOQ fortification of TX control soil, sample 80128-308, the equation for the standard curve of the 296.2/131.1 Da ion transition was:

$y = -7748.66\ x^2 + 879633\ x + 19193.9$

The peak area (y) for the injection was 90565cps. Substituting the peak area (y) into the quadratic equation above and solving for the positive value of x yields

x = 0.0812 ng/mL

as it was calculated by the Analyst software. The concentration value (x) in ng/mL was then multiplied by the analysis volume (10 mL), the aliquot factor (50 mL extraction vol./1 mL aliquot vol.) and divided by the sample mass (5 g) to result in a final concentration of 8.12 ng/g = 8.12 ppb.

x ppb = (0.0.0812 ng/mL) × 10 mL × 50 mL/1 mL ÷ 5 g = 8.12 ng/g = 8.12 ppb

The percent recovery was calculated by dividing the residue found by the actual fortification level:

$$\% \text{ Recovery} = \frac{8.12 \text{ ppb}}{10 \text{ ppb}} = 81\%$$

Note that the numbers calculated by Analyst may differ slightly from those calculated by hand because the slope, intercepts, and quadratic curve coefficients shown on the calibration curve printout are truncated and do not show the same number of digits as used by Analyst in its calculations.

5.0 DISCUSSION AND CONCLUSION

Trial 1 samples were analyzed initially on a Sciex 4000 LCMSMS system. The data from these injections sets were rejected due to the low sensitivity in the negative mode demonstrated by this instrument. (Refer to the Correspondence between the Study Director and the Sponsor Representative dated 28 March 2014). Deep cleaning of that particular instrument and reinjection of extracts confirmed the original results.

The decision was made to inject the Trial 1 samples on a different LC/MS/MS instrument (Sciex 5000). Acceptable recoveries on both soil types and all spiking levels were obtained on this system (see Section 3.5 for Instrument Description and Analytical conditions.) No modifications to the method were necessary for successful validation of Method-80127-000. Therefore, the ILV was successful on the first trial.

6.0 COMMENTS ON THE METHOD

As stated in the previous section, no method modifications were required to yield acceptable results and the successful completion of the ILV study. The method yielded acceptable results as it was written, however a minor change was made to prevent overlap of equipment used by the method developer. A reciprocating shaker was used in place of the Genogrinder™ shaker (see communication 1 dated 7 January 2014.)

Minor typographical errors exist in ABC Method No. M-80127-000 that should be corrected in a subsequent version.

- Section 1.0 of the method should be revised to correct the units of the LOD (approximately 30% of the LOQ) to "ppb."
- In Section 4.0, the formic acid component name of the Extraction Solution should be corrected to "0.1% Formic Acid in (1:1) acetonitrile/water solution."
- Section 6.4.3 should be re-worded to allow a range of steel balls of approximate diameter, for example "add a few (3-5) approximately ¼ inch steel balls and 15 mL of acetonitrile." Adding the steel balls before the solvent will also prevent inadvertent splashing.

Approximately 7.25 person-hours were required to aliquot, fortify, and extract a set of 12 samples, i.e., approximately one business day for one analyst. Instrument analysis of

the Validation samples was based on an injection sequence starting with an injection solvent blank, followed by three Calibration Standard injections, then by five sample injections between Calibration Standard injections, and ending with three Calibration Standard injections. With a per sample run time of approximately 7 minutes, instrument acquisition time is approximately 2.7 hours for a 23-injection run sequence (12 samples + 1 reagent blank + 10 calibration standards.) Therefore, a total of approximately 10 hours were required to complete one set of samples, or one calendar day. Time associated with Standard preparation, laboratory cleanup, data work up, and data checking is not included in this total. An additional 4 hours is approximated for the preparation of standard solutions and laboratory solutions. Data work up and review can be accomplished within four hours for a typical set of samples if quantification of the confirmatory transition is not required.

7.0 PROTOCOL DEVIATIONS

None

8.0 ARCHIVE STATEMENT

Original raw data, including, but not limited to, copies of the original chromatograms, worksheets, correspondence, and results shall be included with the data package submitted by the Test Facility to be archived at:

EPL Archives, Inc.
45604 Terminal Drive
Sterling, VA 20166

Verified copies of the original protocol, amendments, final report, raw data, and all pertinent information will be maintained in ABC's archives. The Test Facility shall keep any instrument, equipment, and storage logs for the lifetime of the product and shall obtain permission of the Sponsor before discarding. The Test Facility shall keep all records as described above along with an electronic copy of these records in its archives until further notice from the Sponsor.

Facility records pertaining to multiple studies (refrigerator/freezer records, equipment logbooks, etc.) are archived at the facility in which they were generated.

9.0 REFERENCES

(1) United States Environmental Protection Agency (U.S. EPA). Office of Chemical Safety and Pollution Prevention, Ecological Effects Test Guidelines OCSPP 850.6100, Environmental Chemistry Methods and Associated Independent Laboratory Validation January 2012.

ABC Laboratories, Inc. #80128

The following is a listing of communications, regarding performance of the method, which took place between the confirmatory laboratory and the Sponsor Representative. Included are reasons for the contact, any changes that resulted, and time of this communication with respect to the progress of the confirmatory trial (i.e., before the first trial, during the first trial, etc.):

1. 07 January 2014: An email message was sent from A.G. Gant to Mark Lenz with questions regarding the use of genogrinders, the size of the steel balls specified in the method, which soil to use in the ILV, and which clarification of regulatory guidelines to be used. Also gave notification that a draft protocol was prepared and pending QA review. Del A. Koch, Senior Program Manager, ABC and Kristen Finley, Associate Scientist II, ABC were copied in the email. (Before first trial)

2. 10 January 2014: An email message was sent from A.G. Gant to Mark Lenz with an attached copy of the draft protocol. Del A. Koch and Kristen Finley were copied in the email. (Before first trial.)

3. 16 January 2014: An email message was sent from Mark Lenz to A.G. Gant advising that he has suggestions and comments for the protocol. (Before first trial.)

4. 16 January 2014: An email message was sent from A.G. Gant to Mark Lenz with questions regarding which control soils to use and acknowledging the correct archive address. (Before first trial.)

5. 16 January 2014: Mark Lenz, Study Monitor, Cheminova, Inc. telephoned A.G. Gant, Study Director, ABC Laboratories, Inc. to discuss the draft protocol and study schedule. (Before first trial.).

6. 21 January 2014: An email message was sent from Mark Lenz to A.G. Gant advising that Cheminova has no comments for the protocol and requesting finalization/signature. (Before first trial.)

7. 22 January 2014: An email message was sent from Mark Lenz to A.G. Gant advising ABC to capture appropriate signatures before starting, and to forward ABC signed protocol for Cheminova signatures. (Before first trial.)

8. 22 January 2014: An email message was sent from A.G. Gant to Mark Lenz advising the he is out of the office, work will begin on Thursday. (Before first trial.)

9. 23 January 2014: An email message was sent from A.G. Gant to Mark Lenz with the ABC signed protocol attached. Paul Whatling, Cheminova, was copied on the email. (Before first trial.)

10. 23 January 2014: Mark Lenz forwarded an email from Paul Whatling advising that the protocol has been reviewed and there are no comments. (Before first trial.)

11. 29 January 2014: An email message was sent from A.G. Gant to Mark Lenz informing him that the test plot information in the protocol is incorrect. A protocol amendment will be prepared. Kristen Finley was copied on the email. (Before first trial.)

12. 31 January 2014: An email message was sent from Mark Lenz to A.G. Gant acknowledging that an amendment may be filed. (Before first trial.)

13. 10 February 2014: A.G. Gant telephoned Mark Lenz to discuss an error found in the Protocol's description of the Iowa control soil's location and to update on procedures scheduled to begin 11Feb14. (Before first trial.)

14. 12 February 2014: An email message was sent from A.G. Gant to Mark Lenz with the protocol amendment attached. Del Koch was copied on the email. (Before first trial.)

15. 14 February 2014: Mark Lenz telephoned A.G. Gant to obtain a status update. (Before first trial.)

16. 24 February 2014: An email message was sent from Mark Lenz to A.G. Gant with the signed protocol amendment attached. Paul Whatling was copied on the email. (Before first trial.)

17. 25 March 2014: An email message was sent from A.G. Gant to Mark Lenz to update the status of the project. Preliminary results showed that ABC did not yet have sufficient sensitivity for one MET-42 transition and was encountering too much response for Pethoxamid. The recommendation was to use the 340/79.9 transition for MET-42 quantification and the 340/120.7 transition as the confirmatory ion. Trial one scheduled for extraction 26Mar14. (Before first trial.)

18. 25 March 2014: An email message was sent from Mark Lenz to A.G. Gant confirming receipt of prior email. (Before first trial.)

19. 28 March 2014: An email message was sent from A.G. Gant to Mark Lenz with an attached Word document summarizing the recoveries from the ILV testing. Signal suppression affected recoveries for MET-42, however Pethoxamid values looked great. Del A. Koch and Kevin Wells, Staff Scientist/Group Leader, ABC were copied in the email. (During analysis of first trial.)

20. 03 April 2014: A.G. Gant telephoned Mark Lenz to discuss instrument conditions and performance related to MET-42 sensitivity drop. Trial 1 extracts will be re-injected following an instrument deep clean, calibration, and re-optimization for MET-42. It was noted that oxidation of the steel balls was apparent in the reagent blank, which may not be present in extracts generated by the Genogrinder shaker. Standard solution was prepared in the reagent blank extract to compare against calibration standard to determine if the 30-40% suppression was related to the presence of ferrous oxide or the soil matrix. (During analysis of first trial.)

21. 08 April 2014: An email message was sent from A.G. Gant to Mark Lenz to report that the testing planned for the weekend did not occur due to IT problems and request feedback on the possibility of discussing the difficulties of the negative ion mode analysis of MET-42 with a developer contact. (During analysis of first trial.)

22. 08 April 2014: An email message was sent from Mark Lenz to A.G. Gant to advise that conducting a deep clean and infuse would be good prior to speaking with developer and to carefully plan and document discussion. Emphasized desire to not go to a second trial. (During analysis of first trial.)

23. 08 April 2014: An email message was sent from A.G. Gant to Mark Lenz to acknowledge that any communication with the developer would occur after the deep clean, MET-42 infusion, and subsequent injection tests. He also expressed a desire to go straight to the MS analyst, and indicated that the developer is correct that the method works well for extraction, but that it may need some provisos with regard to the negative mode. He also states that a second trial will not be needed unless the presence of the ferrous oxide balls is part of the signal suppression. (During analysis of first trial.)

24. 08 April 2014: Mark Lenz replied to A.G. Gant affirmatively. (During analysis of first trial.)

25. 16 April 2014: An email message was sent from A.G. Gant to Mark Lenz to update that no work has been performed due to instrument availability. Del A. Koch, Kevin Wells, and Wes Winberry, Senior Program Manager, ABC were copied in the email. (During analysis of first trial.)

26. 18 April 2014: An email message was sent from A.G. Gant to Mark Lenz to update that the instrument will be available for use and to expect tabulated results Monday evening. Del A. Koch, Kevin Wells, and Wes Winberry were copied in the email. (During analysis of first trial.)

27. 22 April 2014: An email message was sent from Mark Lenz to A.G. Gant to acknowledge updated testing timeline. (During analysis of first trial.)

28. 22 April 2014: An email message was sent from A.G. Gant to Mark Lenz to update that testing was performed, but data has not yet been integrated. Wes Winberry was copied in the email. (During analysis of first trial.)

29. 28 April 2014: An email message was sent from Mark Lenz to A.G. Gant requesting an update. (During analysis of first trial.)

30. 29 April 2014: An email message was sent from A.G. Gant to Mark Lenz to update that testing was performed, but instrument issues were again encountered regarding the MET-42 suppression in the negative ion mode. Testing did reveal that the suppression is not due to oxidation of the steel balls and Trial 2 will not be necessary. (During analysis of first trial.)

31. 29 April 2014: An email message was sent from Del A. Koch to A.G. Gant updating him on a phone conversation he had with Mark Lenz regarding possible use of a Sciex 5000. Mark Lenz and Wes Winberry were copied in the email. (During analysis of first trial.)

32. 6 June 2014: An email message was sent to Mark Lenz from A.G. Gant updating him on the analytical results of the Trial1 samples acquired on the Sciex 5000. Del Koch and Wes Winberry were copied on the email. Mark Lenz replied stating the results looked good. (Following analysis of first trial.)