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Method No.: M-80127	**Version: 001**	**Supersedes: 000**
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Title: Analytical Method for the Determination of Pethoxamid and MET-42 in Soil via LC/MS/MS		

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Revision History:

Version 001: ABC logo was updated
- Section 1.0 "LOD of 3 ppm" was corrected to "LOD of 3 ppb"
- Section 4.0 "0.1% Formic Acid (aq) in (1:1) acetonitrile/water solution" was corrected to "0.1% Formic Acid in (1:1) acetonitrile/water solution"
- Section 6.4.3 "Add 15 mL of acetonitrile (ACN) and three ¼ inch steel balls" was corrected to "Add a few (3-5) approximately ¼ inch steel balls and 15 mL of acetonitrile"

1.0 Objective/Purpose/Principle

This analytical method was developed for the detection, quantification, and confirmation of pethoxamid and MET-42 in soil matrices using LC/MS/MS, based off PTRL Europe Study No. P 1578 G method (See Reference 1). Soil from Iowa, New York, Texas, and California (IA, NY, TX, and CA, respectively) were used in the method development as representative matrices. The method limit of quantitation (LOQ) is 10 ppb with an estimated LOD of 3 ppb (approximately 30% LOQ).

The analytical methodology described herein provides the procedure to determine residues of pethoxamid and MET-42 in soil matrices. It consists of a rigorous high speed shake with steel balls on a Geno/grinder® extraction in the presence of an organic solution, followed by two more shakes in the presence of aqueous/organic solution as indicated in the metabolism study used to support extraction efficiency (See Reference 3) prior to LC/MS/MS analysis.

2.0 Safety Precautions

	Water	Acetonitrile	Formic Acid
Harmful Vapor	×	✓	✓
Flammable	×	✓	×
Harmful by Skin Absorption	×	✓	✓
Irritant to Respiratory System and Eyes	×	✓	✓
Causes Burns	×	×	✓

All materials should be handled in accordance with typical safe-handling procedures. Consultation of the appropriate MSDS is also advised.

3.0 Materials and Equipment

Equivalent glassware and equipment may be substituted where appropriate.

- Hammermill (or another appropriate homogenizing device)
- Geno/grinder® SPEX Sample Prep 2010
- Volumetric glassware, available from Fisher Scientific (Fairlawn, NJ)
- Beakers and measuring cylinders, available from Fisher Scientific (Fairlawn, NJ)
- Pasteur pipettes, disposable, available from Fisher Scientific (Fairlawn, NJ)
- Analytical balance (to 5 figures), available from Mettler-Toledo, Inc. (Columbus, OH)
- Top loading balance, available from Mettler-Toledo, Inc. (Columbus, OH)
- Extraction vessels, polypropylene tubes, 50 mL capacity (Code: 14-959-49A) from Fisher Scientific (Fairlawn, NJ)
- Eppendorf type pipettes and tips, available from Fisher Scientific (Fairlawn, NJ)
- Centrifuge, available from Beckman Coulter (Brea, CA)
- Centrifuge, available from DuPont Instruments (Miami, FL)
- Applied Biosystems/Sciex API 4000 MS/MS with Agilent 1200 HPLC (Agilent, Santa Clara, CA)
- Synergi Polar-RP column, 100A (50 x 3.00 mm, 2.5 micron), Phenomenex

4.0 Reagents and Solution

Equivalent reagents may be substituted for those listed below. To determine if impurities in substituted reagents interfere with analyses, a "reagent blank" should be prepared using appropriate amounts of the solvents, and using the chromatographic conditions specified in this method.

- Acetone (Code: A949-4), available from Fisher Scientific (Fairlawn, NJ)
- Acetonitrile (Code:A996-4), available from Fisher Scientific (Fairlawn, NJ)
- Formic Acid (Code: A117-50), available from Fisher Scientific (Fairlawn, NJ)
- Water (Code: A456-4), available from Fisher Scientific (Fairlawn, NJ)

- Extraction Solution: 0.1% Formic Acid in (1:1) acetonitrile/water solution; Combine 500 mL water, 500 mL of acetonitrile, and 1 mL of concentrated formic acid (98+% pure). Mix well.
- Injection Solution: 0.1% Formic Acid in 30:70 Acetonitrile/water solution; Combine 700 mL purified water, 300 mL acetonitrile, and 1 mL of concentrated formic acid (98+% pure). Mix well.
- Mobile Phase A: 0.1% Formic Acid (aq) solution; Add 1 mL of concentrated formic acid (98+% pure) to 1 L of purified water. Mix well.
- Mobile Phase B: 0.1% Formic Acid in Methanol solution; Add 1 mL of concentrated formic acid (98+% pure) to 1 L of methanol. Mix well.

5.0 Reference Items for Calibration and Fortification

Reference analytical standards were obtained from Cheminova A/S P.O. Box 9, DK-7620 Lemvig, Denmark.

Compound	Ref No.	Purity (%)
Pethoxamid	460-02	99.8 (w/w)
Met-42	507-01	93.3 (w/w)

5.1 Fortification Solutions and Calibration Solutions

Stock Standard Solution for Pethoxamid at 1.00 mg/mL

Prepare stock standard solution by weighing 10 mg (adjusted for purity) of pethoxamid analytical standard into a 10-mL volumetric flask using an analytical balance. Dissolve the standard in acetone and mix thoroughly.

Stock Standard Solution for MET-42 at 1.00 mg/mL

Prepare stock standard solution by weighing 10 mg (adjusted for purity) of MET-42 analytical standard into a 10-mL volumetric flask using an analytical balance. Dissolve the standard in acetone and mix thoroughly.

10,000 ng/mL Intermediate Standard Solution Combine

Prepare intermediate standard solution by transferring 0.1 mL of 1.00 mg/mL stock standard solution of each analyte into a 10-mL volumetric flask and diluting to volume with 0.1% formic acid in (30:70) acetonitrile/water solution. Mix thoroughly.

1,000 ng/mL Fortification Solution

Prepare a fortification solution by transferring 1.00 mL of 10,000 ng/mL intermediate standard solution to a 10-mL volumetric flask and diluting to volume with 0.1% formic acid in (30:70) acetonitrile/water. Mix thoroughly.

100 ng/mL Fortification Solution

Prepare a fortification solution by transferring 1.00 mL of 1,000 ng/mL fortification solution to a 10-mL volumetric flask and diluting to volume with 0.1% formic acid in (30:70) acetonitrile/water. Mix thoroughly.

The stability was observed to be approximately 30 days. These standards will be used for fortifications and for preparation of injection/calibration standards. The preparation of these standard solutions may be achieved by the use of alternative dilutions as necessary, and alternate concentrations may be used as appropriate to the analysis.

Pethoxamid and MET-42 Calibration Solutions

A range of pethoxamid and MET-42 calibration solutions should be prepared in a similar manner as the fortification standards above (serial dilutions) using 0.1% formic acid in (30:70) acetonitrile/water solution. One possible dilution scheme follows:

Initial Std ng/mL	Aliquot Volume mL	Final Volume mL	Calibration Solution
1,000	0.500	10	50.0
1,000	0.250	10	25.0
100	1.00	10	10.0
50.0	1.00	10	5.00
25.0	1.00	10	2.50
10.0	1.00	10	1.00
5.00	1.00	10	0.500
2.50	1.00	10	0.250
1.00	1.00	10	0.100
0.500	1.00	10	0.050

The above scheme uses 10 mL volumetric glassware and Eppendorf-type micropipettes to dispense the required volumes. They should be stored at or below 4°C and are good for at least 30 days. Longer storage intervals may be assessed at the discretion of the analyst.

6.0 Specimen Preparations and Extractions

Control materials used for method development were obtained from IA, NY, TX and CA.

6.1 Soil Preparation

Soil samples should be frozen prior to processing. A hammermill, or another appropriate device, is used to process the soil into a homogenous powder. Place the homogenized soil in a labeled plastic container in a freezer and allow the dry ice to dissipate. Keep the soil samples frozen until needed. Mix the sample thoroughly during grinding to obtain a homogenous mixture.

6.2 Controls and Reagent Blank

At least one untreated control sample must be analyzed with each set of samples to ensure that no contamination of the samples has occurred prior to, or during, the analysis from matrix, solvents, or materials. A reagent blank may also be included in a batch if deemed necessary.

6.3 Fortification Samples

Appropriately fortified control samples should be analyzed with each set to assess the performance of the method. Typically, two procedural recoveries (i.e. fortified control samples) will be run; one of these will usually be fortified at the Limit of Quantitation (LOQ) and the other at 10 times the LOQ (or at a higher level if this is anticipated in the samples).

6.4 Extraction

See Appendix 1 for schematics of the methods. Step-by-step directions are given below.

6.4.1 Weigh 5.0 g (± 0.05 g) of the sample into a 50-mL polypropylene centrifuge tube.

6.4.2 Fortify any procedural recoveries at this stage by spiking control sample with the appropriate amount of reference material. For example, to fortify at 10 ppb (LOQ), accurately add 0.50 mL of a 100 ng/mL solution of pethoxamid and MET-42 to a 5.0-g sample. Other examples are shown below.

Fortification level (ppb)	Weight of sample (g)	Fortification solution (ng/mL)	Volume used (mL)
10	5.0	100	0.50
300	5.0	10,000	0.15

6.4.3 Add a few (3-5) approximately ¼ inch steel balls and 15 mL of acetonitrile.

6.4.4 Place sample into a Geno/grinder® and homogenize for 2 minutes at ~1200 strokes/minute.

6.4.5 Then centrifuge the samples at ~ 3000 RPM for ~5 minutes to form a pellet.

6.4.6 Decant the supernatant of the sample into clean 50-mL polypropylene centrifuge tube.

6.4.7 Add 15 mL of 0.1% formic acid in (1:1) ACN:water (H_2O) to the extraction tube.

6.4.8 Tap and shake until the pellet is well disperse into the solution.

6.4.9 Place the sample into a Geno/grinder® and homogenize for 2 minutes at ~1200 strokes/minute.

6.4.10 Centrifuge the sample at ~ 3000 rpm for ~5 minutes to form a pellet.

6.4.11 Decant the supernatant into the 50 mL centrifuge tube from step 6.4.1.6

6.4.12 Repeat steps 6.4.7, to 6.4.11 then adjust the final extraction volume to 50 mL with 0.1% formic acid (aq) and vortex the sample.

6.4.13 Aliquot 1 mL of the extract into a 15 mL polypropyle. e tube and dilute to 10 mL with 0.1% formic acid in (30:70) ACN : H_2O

6.5 Dilutions

If dilutions are required, they should be made with 0.1% formic acid in (30:70) acetonitrile/water and diluted within the curve range.

6.6 Method Stopping Points

An analyst should be able to complete the method in an eight hour work day with injections performed by an automated system overnight. Acceptable method recoveries will validate any work flow interruptions. Sample extracts should be stored refrigerated in sealed containers.

6.7 Method Validation

This method has been GLP validated on four soil types at ABC Laboratories, Inc., under ABC study 80073 (See Reference 2).

7.0 Instrumentation

This method uses a gradient-elution, reversed-phase HPLC analysis on a Synergi Polar-RP column. The column choice reflects experimental results indicating optimum chromatographic separation from co-extractants. Two transitions are monitored in this method as a confirmation of analyte detection. Alternative chromatographic conditions can be used provided the analytical method is validated and acceptable recoveries are obtained.

System:	Applied Biosystems/Sciex API 4000 MS/MS with Agilent System with Pal Auto-sampler
Column:	50 × 3.0 mm, Synergi Polar-RP analytical column with 2.5-µm diameter packing.
Column Temperature:	40°C
Fixed Loop:	10 µL
Sample syringe size:	100 µL
Autosampler Temperature:	10°C
Flow Rate:	No Split
Conditions:	Time %A %B Flow Rate (mL/min) 0.00 40.0 60.0 0.500 2.50 15.0 85.0 0.500 4.00 15.0 85.0 0.500 4.10 40.0 60.0 0.500 6.00 40.0 60.0 0.500 Mobile Phase A: 0.1% Formic Acid in Water Mobile Phase B: 0.1% Formic Acid in Methanol
Approximate Retention Times	(minutes)
Pethoxamid and MET-42	2.8 and 0.9
Total Run Time:	6.00 minutes

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)	TEM (°C)	IHE	IS (V)	CAD (PSI)	DP (V)	EP (V)	CE (V)	CXP (V)
Pethoxamid	296.2	131.1	300	50	50	50	500	on	3500	8	20	10	29	15
	296.2	250.2											18	15
MET-42	340.0	120.7	300	50	50	50	500	on	-3500	8	-50	-10	-33	-15
	340.0	79.9											-60	-15

NOTE: *Due to instrument variances, the parameters (i.e. rate and hold times) should be optimized for each instrument. The above conditions were used in the validation sets in ABC-80073* (See Reference 2) *and are presented as a guide.*

8.0 Calibration Procedure and Analysis

A chromatographic standard should be analyzed prior to the start of analyses to establish that the instrument is working properly. Operating parameters must be tailored to the particular instrument used, especially if it is to be an alternate vendor's instrument, and should be checked daily. Note that it may be necessary to monitor alternate ion channels than those chosen during the development of this method when utilizing alternate instrumentation than specified herein. Each ion channel used for sample analysis/ quantitation must be checked to insure it is free of interference. A control will be used to demonstrate that baseline interference is less than signal-to-noise 3:1. Each injection set should start with a standard and end with a standard. Calibration standards should be interspersed among the samples as they are injected. Although instrument dependent, it is suggested that no more than 5 samples are injected between standard injections.

9.0 Calculations

Results may be calculated using a standard curve (linear, weighted, or polynomial) or by using average response factor. If the average response factor method is, the overall relative standard deviation of the standards should be ≤20%. One method of quantitation should be used for quantitation of all analytes throughout a study.

The results for this method were calculated using a linear 1/x weighted curve.

The following equation can be rearranged and used to calculate residues as follows:

$$y = mx + b$$

Where y is the instrument response value, x is the standard concentration, m is the gradient (slope) of the line of best fit and b is the intercept value. An example of this equation generated using the experimental values of m and b should be included in the raw data, as should the "R-Squared" value for the regression.

Re-arrangement for x gives

$$x = \frac{y - b}{m}$$

The concentration (ng/g or ppb) of analyte found in each sample was calculated as follows:

ng/g analyte Found =

$$\frac{[(\text{Peak Area} - b) / m] \times [\text{Aliquot Factor} \times \text{Final Vol. (mL)} \times \text{Dilution Factor}]}{\text{Sample Wt. (g)}}$$

Where:

Total Extract Volume	=	50 mL
Aliquot Taken	=	1.0 mL
Aliquot Factor	=	Total Extract Volume / Aliquot Taken = 50
Final Volume	=	10 mL
Sample Weight	=	5 grams
Dilution Factor	=	Factor of Dilution of Final Volume prior to LC/MS/MS analysis

The percent recovery found was calculated as follows:

$$\%\ Recovery = \frac{(ng/g\ found)}{(Fortification\ level, ng/g)} \times 100\%$$

10.0 References

1. Beck, Iris-Constanze., Validation of Analyical Method(s) for Confirmation of Resdiues of Pethoxamid in Soil and in Water, PTRL No. P 1578G Stahler International GMbH &Co.KG, D-21683 Stade, Germany

2. Vincent, Tim, "Terrestrial Field Dissipation of Pethoxamid at Four Sites, USA," Ongoing study, ABC Study No. 80073, ABC Laboratories, Inc., Columbia, Missouri 65202

3. Monograph. (16 August 2002). *Pethoxamid, Volume I, Report and Proposed Decision*. Rapporteur Member State: Germany.

Appendix 1 Method Schematic

Extraction: