~~MATERIAL AND METHODS~~

Reference/Test Substance

Name:	Chlorpropham (PIN-NIP® 98%)
CAS Name:	1-methylethyl (3-chlorophenyl)carbamate
Purity:	99.47%
Supplier:	Wildlife International
Lot no.:	1364
Molecular formula:	$C_{10}H_{12}NO_2Cl$
Molecular weight:	213.7 g/mole
Structure:	
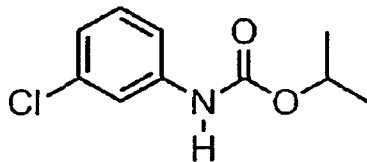
Origin of Reference/Test Substance

The reference/test substance identified as Chlorpropham (lot no. 1364) was provided by Wildlife International and received at PTRL West on March 13, 2014. Upon receipt at PTRL West, the reference/test substance was given the PTRL inventory no. 2574W-001. The reference/test substance was maintained at room temperature when not in use. The Certificate of Analysis is provided in Appendix B.

Solvents/Reagents

Acetonitrile, HPLC grade
Water, HPLC grade
Formic acid

Equipment/Materials List

Laboratory balances
Weighing boats
Pipetmen with plastic disposable tips (adjustable volume pipetors)
Plastic graduated centrifuge tubes (15mL and 50 mL capacity)
Branson sonicator
Sorvall RT7 Plus centrifuge
Whatman nylon syringeless filters (0.2 µm)
Wrist-Action™ shaker
Vortex mixer
GC vials

Glassware:
    Pasteur pipettes
    Graduated cylinders
    Beakers
    Volumetric flasks
    Amber bottles and vials with Teflon® lined caps

Liquid Chromatography/Mass Spectrometry (LC/MS):
Agilent 1200 Series Binary Pump, Model G1312A
Agilent 1200 Series Autosampler, Model G1367B
Agilent 1200 Series Vacuum Solvent Micro-Degasser, Model G1379B
Agilent 1200 Series Column Compartment, Model G1316A
Applied Biosystems MDS/SCIEX API 3200 Mass Spectrometer
Analyst Data System Software

Test Method

The analytical method for the analysis of chlorpropham was validated at PTRL West by Liquid Chromatography with Tandem Mass Spectrometry Detection (LC-MS/MS) based on the analytical method (Reference 1), provided by the sponsor.

The soil samples were spiked with known concentrations of chlorpropham. The samples were extracted twice with ACN followed by a third extraction with ACN:water (75:25). Samples were centrifuged at 4,000 rpm for 10 minutes after each extraction and the combined supernatant was adjusted to 75 mL with ACN. Aliquots of the final extracts (approximately 3 – 5 mL) were filtered through 0.2 μm nylon syringeless filters and a 10 fold dilution of the filtered extracts was prepared in ACN:water (45:55, v/v) and analyzed by LC-MS/MS.

The percent recovery was determined using external standardization where a linear curve for each transition (m/z 172 and 154) was analyzed along with the samples.

Test System

Source and Characterization of Test System

Well soil was collected and characterized by Agvise Laboratories, Inc. (604 Highway 15 West, Northwood, North Dakota) under a related study "Validation of a Method for the Determination of Chlorpropham in Soil for Support of Terrestrial Field Dissipation Studies", (Reference 1). Upon arrival at PTRL West the soil was assigned the inventory no. 2602W-001. The soil sample was stored refrigerated (typically < 4°C) in the dark when not in use.

Preparation of Chlorpropham Stock Solution

A stock solution containing chlorpropham was prepared by weighing an aliquot of the reference substance (54.79 mg) in a weighing boat and transferring into a 50 mL volumetric flask. The stock solution was dissolved and diluted to the mark with ACN. An additional volume of ACN (3.69 mL) was added into the flask to yield a nominal concentration of 1,015 μg/mL. The concentration of the stock solution was corrected for the purity of the reference substance (99.47%). The stock solution was transferred into an amber bottle and stored frozen (typically < -4°C) when not in use.

Preparation of Chlorpropham Intermediate Solutions

Intermediate solutions were prepared in 10 mL volumetric flasks by measuring aliquots of chlorpropham stock solution (1,015 µg/mL) and in serial dilution as follows:

Solution used	Aliquot soln (mL)	Final volume (mL)	Theoretical conc. (µg/mL) [1]	Sample ID
1,015 µg/mL	1.0	10	102	102 µg/mL
102 µg/mL	1.0	10	10	10 µg/mL
10.2 µg/mL	1.0	10	1.0	1 µg/mL
1 µg/mL	1.0	10	0.1	0.1 µg/mL

[1] Theoretical conc. (µg/mL) = [theoretical conc. soln used x aliquot (mL)] ÷ final volume (mL)

Adjustable volume pipetors with plastic disposable tips were used for measuring aliquots. Final solutions were diluted to the mark with ACN. Intermediate solutions were vortexed to mix, transferred into amber bottles and stored frozen (typically < -4°C) when not in use.

Preparation of Chlorpropham Solvent Based Calibrants

Six calibrants were prepared by transferring an appropriate volume of the chlorpropham intermediate solutions via adjustable volume pipetors with plastic disposable tips into separate 10 mL volumetric flasks and diluting to the mark with ACN:water (50:50). Calibrants were stored refrigerated (typically < 10°C) when not in use.

intermediate soln used	Aliquot soln (mL)	Final volume (mL)	Theoretical conc. (ng/mL) [2] Chlorpropham
0.1 µg/mL	0.200	100	0.2
0.1 µg/mL	0.500	100	0.5
0.1 µg/mL	1.000	100	1.0
1 µg/mL	0.250	100	2.5
1 µg/mL	0.500	100	5.0
1 µg/mL	1.000	100	10.0

[2] Theoretical conc stds (ng/mL) = {[theoretical conc sol used (µg/mL) x aliquot (mL)] ÷ final volume (mL)} x 1,000 ng/µg

Preparation of Chlorpropham Fortification Solutions

Fortification solutions were chlorpropham intermediate solutions 10 µg/mL (for LOQ) and 102 µg/mL (for 10X LOQ) described in the previous section above.

Fortification Procedure

Fortification of untreated soil samples was conducted at two fortification levels as shown below:

Fortification Level (mg/kg)	Chlorpropham
0.05	0.05 mL of 10 µg/mL in 10 g soil
0.5	0.05 mL of 102 µg/mL in 10 g soil

Fortification was conducted to determine the percent recovery within the method validation. This procedure was performed in quintuplicate during method validation at each fortification level.

Extraction Method

1. Weigh 10 g ± 0.1 g aliquots of soil in a 50 mL plastic graduated centrifuge tube.

2. Fortify as necessary using a 200 µL adjustable volume pipetor with plastic disposable tips.

3. Extract with 25 mL ACN, sonicate for 5 minutes, and centrifuge at 4,000 rpm for 10 minutes.

4. Decant soil extract into a 100 mL glass graduated cylinder.

5. Extract soil pellet with 25 mL ACN without sonication, shake on Wrist-Action™ shaker for 15 minutes, and centrifuge as in step 3.

6. Combine soil extracts in the same graduated cylinder.

7. Extract third time with 25 mL ACN:water (75:25), shake and centrifuge as described in steps 3 and 5.

8. Combine all soil extracts in the graduated cylinder and adjust volume to 75 mL with ACN.

9.   Transfer combined soil extract into an amber bottle and mix.

10.   Filter aliquot (~3 – 5 mL) of soil extract and through a 0.2 μm nylon syringeless filter.

11.   Volumetrically measure an aliquot (1 mL) of filtered soil extract via a 1 mL pipetman with disposable plastic tip and transfer into a 15 mL plastic graduated centrifuge tube.

12.   Adjust volume to 10 mL with ACN:water (45:55, v/v) and vortex to mix.

13.   Analyze samples by LC-MS/MS.

14.   Remaining extracts are transferred into amber bottles and stored frozen (typically < -4°C) when not in use.

A schematic diagram of the extraction method is presented in Figure 1.

Liquid Chromatography with Tandem Mass Spectrometry Analytical Method (LC-MS/MS)

LC conditions

Column: ACE Excel 2 C18-AR 2 μ, 50 mm x 2.1 mm
Guard column: Phenomenex C18, 4 mm x 2 mm (AJO-4286)
Column Temperature: 40°C
Injection Volume: 50 μL
Mobile Phase System: A)   0.1 % formic acid in HPLC grade Soil
                     B)   0.1% formic acid in ACN

Gradient:

Time (min.)	Flow Rate (μL/min.)	% A	% B
0.0	350	80	20
1.0	350	80	20
4.0	350	5	95
6.0	350	5	95
6.1	350	80	20
10.0	350	80	20

Diverter Valco Valve: at 3.0 minutes to MS, at 6.2 minutes to waste

Approximate retention time:
- Chlorpropham: 4.7 min

MS conditions

Electrospray Ionization Mass Spectrometry:

An Applied Biosystems MDS/SCIEX API 3200 tandem mass spectrometer was used with electrospray ionization (ESI) in positive polarity mode to acquire data by Multiple Reaction Monitoring (MRM).

Period 1 settings: Experiment 1:

Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	CE	CXP
214.0	172.0	200	14	2.5
214.0	154.1	200	25	2.4
214.0	126.0	200	35	2.0

Representative Mass Spectrometer Settings:

	Period 1
Scan Type:	MRM
Polarity:	Positive
Ion Source:	Turbo Spray
CAD:	5.0
CUR:	30.0
GS1:	90.0
GS2:	30.0
IS:	5500.0
TEM:	500.0
Ihe:	ON
DP:	18.0
EP:	5.0
CEP	18.39

Note: the full scan (212 – 216 amu) and product ion scan (MS/MS) for chlorpropham are provided in Figures 2 and 3 respectively. Based on the product ion scan, m/z ion 172 was considered as the quantitation ion (most abundant ion) and m/z 154 was considered as the confirmation (qualifier) ion.

LC-MS/MS Analysis

Samples were analyzed interspersed between the calibrants. Calibrants and samples were analyzed in single injections. ACN:water (1:1. v/v) was analyzed as the solvent blank.

A standard solution (2.5 ng/mL) was reanalyzed as check standard (quality control standard) at the end of the sequence to ensure good chromatography and consistent instrument performance. The stability of the signal was monitored by comparing the response (chlorpropham peak area) of a quality control standard injection with that of a comparable standard from the linear curve within the sequence.

The injection sequence for the method validation sample set was: ACN:water (1:1) (solvent blank), 0.2 ng/mL calibrant, 0.5 ng/mL calibrant, ACN: soil (1:1) (solvent blank), reagent blank, control sample 1, control sample 2, 1 ng/mL calibrant, fortified sample A1, fortified sample A2, fortified sample A3, 2.5 ng/mL calibrant, fortified sample A4, fortified sample A5, fortified sample B1, 5 ng/mL calibrant, fortified sample B2, fortified sample B3, 10 ng/mL calibrant, fortified sample B4, fortified sample B5, 10 ng/mL QC standard, 10 ng/mL QC standard.

Methods of Calculation

Preparation of Stock Standard Solutions

$$\text{Volume of solvent (mL)} = \frac{(W) \times 1000\,\mu g/mg \times (P)}{(FC)}$$

where   W = Milligrams of neat standard
               P = Chemical purity of neat standard
               FC = Final Concentration ($\mu g/mL$)

Quantitation

Separation of chlorpropham was achieved by LC. The detection was by LC-MS/MS with electrospray ionization (ESI) in positive polarity mode. The target analyte was identified by the coincidence of its retention time with its reference substance and MS characteristics. The quantitation of chlorpropham was determined by peak area relative to the concentration of the calibrants. The content of chlorpropham in samples was

quantitated against a 1/x weighted linear curve (y = mx + b) of each transition ion from chlorpropham calibrants where:

y = peak area
x = ng/mL analyte
m = slope
b = intercept

The calculation of weighted curve equation (linear regression) and concentration (ng/mL) present in samples and calibrants was conducted using Analyst software. The amount of chlorpropham was determined for the quantitation ion at m/z 172 and for the confirmation ion at m/z 154.

Recoveries from fortified samples were determined by calculating the found concentration and dividing by the relevant fortification level.

Residue in soil

Found/detected conc (mg/kg chlorpropham) =

$$\frac{\text{calculated conc (ng/mL) of chlorpropham x dilution factor x extraction volume x 1,000 g/kg}}{1{,}000{,}000 \text{ ng/mg x sample weight}}$$

Where:

Dilution factor = (10 mL final volume ÷ 1 mL aliquot) = 10
Extraction volume (mL) = 75
Sample weight (g) = 10

Example: Fortified sample FA1 (m/z 172)

$$\text{mg/kg chlorpropham} = \frac{0.6640 \text{ ng/mL x 10 x 75 mL x 1,000 g/kg}}{1{,}000{,}000 \text{ ng/mg x 10 g}} = 0.0498$$

Percent recovery of chlorpropham in soil

$$\% \text{ Recovery} = \frac{\text{mg/kg detected - mg/kg Control}}{\text{Fortification Level (mg/kg)}} \times 100$$

Example: Fortified sample FA1 (m/z 172)

$$\% \text{ Chlorpropham} = \frac{0.0498 \text{ mg/kg}}{0.05 \text{ mg/kg}} \times 100 = 100\%$$

No chlorpropham residue was detected in the controls

Transcriptions (spreadsheets) of the raw data to support calculations for this study are presented in Appendix C.

Limit of Detection

The limit of detection was 0.015 mg/kg in soil as defined by the lowest calibrant (0.2 ng/mL).

Limit of Quantitation

The limit of quantitation was assigned as the lowest fortification level of chlorpropham validated by the analytical method. The LOQ for chlorpropham in soil was 0.05 mg/kg.

Time Required for Completion of a Sample Set

A sample set consisted of thirteen samples which was comprised of five fortified soil samples at each level (LOQ and 10X LOQ), two controls (untreated soil sample), and one reagent blank. Time required per sample set from initiation of extraction until the completion of instrumental analysis and data evaluation by one analyst is as follows:

- Sample preparation (fortification and dilution) takes approximately 3.5 hours

- LC-MS/MS analysis and data processing (two transition ions) take approximately 1.5 hours

TOTAL = approximately 5 hours for one analyst to complete a sample set. This does not include preparation of calibrants and fortification solutions.

Modifications of the Analytical Method Validation

The analytical method was run exactly as written in Wildlife report (reference 1) with minor modifications:

- The first extraction of the soil extracts was carried out with a Branson sonicator for 5 minutes instead of a Bransonic ultrasonic disruption sample processor at an amplitude setting of approximately 45%.

- Samples were shaken using a Wrist-Action [TM] shaker instead of a gyratory shaker table apparatus.

- Extracts were centrifuged at 4,000 rpm for 10 minutes instead of 4,500 rpm for approximately 5 minutes.

- An equivalent LC column (ACE Excel 2 C18-AR 2 μ, 50 x 2.0 mm) with guard column (Phenomenex C18, 4 mm x 2 mm, AJO-4286) were used for analysis instead of THERMO EC Betasil C-18 (50 mm x 2.1 mm, μm) with guard column (THERMO EC Betasil C-18 (20 mm x 2.1 mm).

No impact on the study was observed.

Statistical Methods

Means, standard deviation, relative standard deviation, and 1/x linear regression were the only statistical methods employed in this study.

Figure 1.   Schematic Diagram of the Analytical Method.

Weigh
10 g +/- 0.1 g soil   50 mL plastic graduated centrifuge tubes

Fortify   as necessary (200 μL adjustable volume pipetor with plastic disposable tips)

Extraction

1. First extraction: 25 mL ACN; sonicate for 5 min; centrifuge 4,000 rpm/10 min
2. Decant soil extract into a 100 mL glass graduated cylinder
3. Second extraction: 25 mL ACN; shake for 15 min on Wrist Action Shaker; centrifuge as step 1
4. Combine soil extracts in same glass cylinder
5. Third extraction: 25 mL ACN: water (756:25); shake, centrifuge and combine soil extract as steps 3, 1, and 4
6. Adjust final volume to 75 mL with ACN
7. Transfer soil extract into amber bottle and m ix

Filtration   8. Filter aliquot (~ 3 -5 mL) of soil extract through a 0.2 μm nylon syringeless filter

Dilution   9. Volumetrically measure 1 mL of filtered soil extract into a 15 mL plastic graduated centrifuge tube
10. Adjust volume to 10 mL ACN:water (45:55, v/v) and vortex to mix

LC-MS /MS
analysis