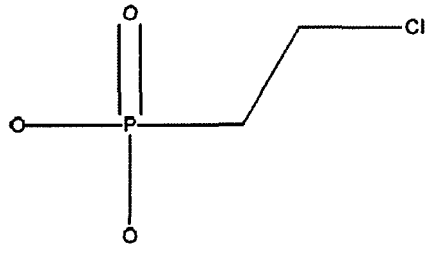
**Retention of Samples**

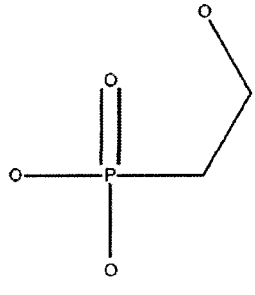
None necessary

**Analytical Reference Standards**

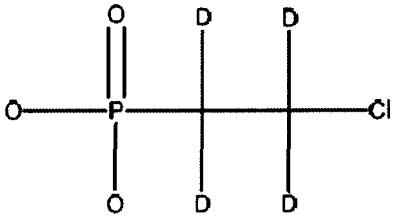
| | |
|---|---|
| Standard Name: | Ethephon |
| Standard Number: | K-1826 |
| IUPAC Name: | (2-Chloroethyl)phosphonic acid |
| CAS Number: | 16672-87-0 |
| Reference Substance Lot: | 0627200304 |
| GLP Purity: | 95.9% |
| Molecular Formula: | $C_2H_6ClO_3P$ |
| Average Mass: | 144.49 |
| Molecular Structure: | |



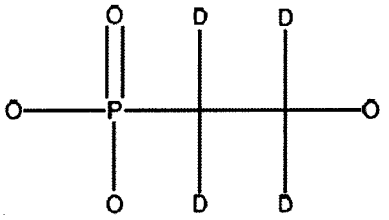

| | |
|---|---|
| Standard Name: | 2-HEPA |
| Standard Number: | K-2074 |
| IUPAC Name: | (2-Hydroxyethyl)phosphonic acid |
| CAS Number: | 22987-21-9 |
| Reference Substance Lot: | 0714200415 |
| GLP Purity: | 95.3% |
| Molecular Formula: | $C_2H_7O_4P$ |
| Average Mass: | 126.05 |
| Molecular Structure: | |



**Internal Standard (IS)**

| | |
|---|---|
| Internal Standard Name: | *d4*-Ethephon |
| Standard Number: | K-1379 |
| IUPAC Name: | (2-Chloroethyl-*1,1,2,2-d4*) phosphonic acid |
| Reference Substance Lot: | 0827200401 |
| GLP Purity: | 0.46% |
| Molecular Formula: | $C_2H_2D_4ClO_3P$ |
| Average Mass: | 148.52 |
| Molecular Structure: |  |



| | |
|---|---|
| Internal Standard Name: | *d4*-HEPA |
| Standard Number: | K-2080 |
| IUPAC Name: | (2-Hydroxyethyl-*1,1,2,2-d4*) phosphonic acid |
| Reference Substance Lot: | NA |
| GLP Purity: | 97.0% |
| Molecular Formula: | $C_2H_3D_4O_4P$ |
| Average Mass: | 130.07 |
| Molecular Structure: |  |



**Other**

Upon completion of the study, a copy of the protocol and the final report will be archived at CPS. The original protocol, final report, raw data, correspondence, and other documentation will be transferred to the Bayer CropScience Archives, Bayer CropScience, 2 T.W. Alexander Drive, RTP, North Carolina, 27709.

# 3.0 MATERIALS AND METHODS

## 3.1 Test Substance

| | |
|---|---|
| **Standard name:** | Ethephon |
| **Standard No.:** | K-1826 |
| **IUPAC name:** | (2-Chloroethyl)phosphonic acid |
| **CAS number:** | 16672-87-0 |
| **Ref. Substance Lot:** | 0627200304 |
| **GLP purity:** | 95.9% |
| **Expiration date:** | 02 Mar 2014 |
| **Storage conditions:** | Freezer |

| | |
|---|---|
| **Internal Standard name:** | *d4*-Ethephon |
| **Standard No.:** | K-1379 |
| **IUPAC name:** | (2-Chloroethyl-*1,1,2,2-d*$_4$)phosphonic acid |
| **Ref. Substance Lot:** | 0827200401 |
| **GLP purity:** | 0.46% |
| **Expiration date:** | 27 Aug 2014 |
| **Storage conditions:** | Freezer |

## 3.2 Test System

The test system used for the validation was a soil sample provided by Bayer. The sample was held at room temperature until needed for analysis.

## 3.3 Equipment and Reagents

The equipment and reagents used for the method validation were as outlined in Bayer Method ET-001-S13-01 for all Trials (Appendix 5, Section 3: Apparatus and Section 4: Reagents and Consumables). Identical or equivalent apparatus and materials were used.

### 3.3.1 Equipment and Apparatus

Milestone ETHOS EX Microwave Extraction System
Beckman Coulter Microcentrifuge (Microfuge 16)
Thermo Megafuge 11R Centrifuge
Volumetric flasks, glass class A (assorted volumes)
Various volume of Manual Micro Pipettor
FisherBrand glass jar with polyvinyl lined cap
Organomation Associates Nitrogen Evaporator
Waters OASIS SPE cartridges (MAX and MCX)

Negative pressure manifold
Vortex mixer
Ultrasonic Cleaner (Branson)
Phenomenex® Aqua C18 150 × 4.6 mm, 3 µm particle size HPLC column
LC-MS/MS – Agilent 1200® binary pump HPLC system with an autosampler (Agilent Technologies) coupled to API 4000™ Tandem Mass Spectrometer with an electrospray ionization interface (Applied Biosystems™)
Various general laboratory glassware and utensils

### 3.3.2 Reagents

Water (EMD)
Methanol (EMD)
Acetonitrile (EMD)
Formic acid (Fluka)
Phosphoric acid (EMD)

## 3.4 Experimental Design

### 3.4.1 Establishment of the Method

Prior to performing the ILV, the analyte retention times, instrument detection limits, and linearity of instrument responses to a range of analyte concentrations were determined, and the test system was verified as free of interferences at appropriate retention times.

### 3.4.2 Sample Validation Sets, Fortification, and Extraction Procedure

Sample Validation Sets
Each analytical set consisted of 13 samples: one reagent blank, two untreated controls, five untreated controls fortified with test substance (ethephon) at the LOQ (5 ppb), and five untreated controls fortified with test substance (ethephon) at 10×LOQ (50 ppb). Internal standard (IS; $d_4$-ethephon) was added to all samples after being extracted at the concentration of 10.0 ng/mL in the final sample solution.

Data for ethephon validation Trial 1 are summarized in Table 1. Residue data sheets are included in Appendix 1.

Calibration standard solutions (0.800 to 100 ng/mL for ethephon, and 10.0 ng/mL for IS), blanks (with IS concentration of 10.0 ng/mL), and double blanks were also included in each sample set.

Fortification
The control LOQ and 10×LOQ samples were fortified with 0.100 mL of the appropriate fortification standard solutions of ethephon (ETH-02 and ETH-03). The fortification standard solutions had a concentration of 1.00 µg/mL (ETH-03) for the LOQ and a concentration of 10.0 µg/mL (ETH-02) for the 10×LOQ.

Extraction and Workup
The following extraction steps were followed for each sample.

1. Transferred 20 ± 0.05 g of soil (for each sample, except for the reagent blank) into a 125-mL (or 250-mL) glass jar containing a magnetic stirbar.
2. Fortified the recovery samples at the desired fortification level with the appropriate fortification standard solutions.
3. Added 40 mL of 0.7% phosphoric acid in water to each sample.
4. Placed jars with soil-solvent mixture (only solvent for the reagent blank sample) into the microwave extractor.
5. Switched on the magnetic stirrer and extracted for 3 minutes at 250 W.
6. Added 0.40 mL of the 1.00 µg/mL $d_4$-ethephon internal standard solution to each sample. Mixed well.
7. Transferred about 1.5 mL of the extract (except for the reagent blank sample, which directly went to Step 8) into a centrifuge tube. Centrifuged for 5 minutes at 10000 rpm to remove fine particles of soil.
8. Transferred an aliquot to an autosampler vial for LC-MS/MS analysis.

### 3.4.3 Sample Processing and Analysis

The samples were processed and analyzed as described by Bayer Method ET-001-S13-01 [1] for all trials, without any major modifications.

### 3.4.4 Fortification and Calibration Standard Solutions Preparation

The primary stock solution for the reference standard and for the internal standard was prepared separately in a 100-mL volumetric flask by dissolving each pre-weighed standard with the appropriate solution. Ethephon (10.23 mg, 95.9% purity) and $d_4$-ethephon (1130.48 mg, 0.46% purity) were dissolved in 0.7% phosphoric acid in water.

Intermediate stock solutions were prepared by diluting ethephon and $d_4$-ethephon primary stock solutions in 0.7% phosphoric acid in water. Two concentration levels (10.0 µg/mL and 1.00 µg/mL) of intermediate stock solutions were prepared for the reference standard and for the internal standard.

The intermediate stock solutions for ethephon, 1.00 µg/mL and 10.0 µg/mL solutions, were used as the fortification solutions for the LOQ and 10×LOQ fortifications, respectively. The

1.00 µg/mL intermediate stock solution for internal standard ($d_4$-ethephon) was added to samples after the sample extraction before the LC-MS/MS analysis.

Calibration standard solutions for ethephon were prepared at concentrations ranging from 0.800 to 100 ng/mL. The calibration standards were prepared by adding appropriate amount of appropriate intermediate stock solutions (including the appropriate IS intermediate stock solutions) to a 50-mL volumetric flask and diluting to volume with 0.7% phosphoric acid in water.

All solutions were stored were refrigerated (4°C) when not in use.

### 3.5 LC-MS/MS Instrumentation

Agilent 1200® HPLC System
ABSciex API 4000 LC-MS/MS
Software: ABSciex Analyst Software, Analyst® 1.5.1
HPLC column: Phenomenex® Aqua C18, 150 mm × 4.6 mm 3 µm particle size

### 3.6 Data Acquisition and Reporting

Peak integration was performed by Analyst® software version 1.5.1. The MS detector responses (peak area) for various injected standard concentrations were used to generate an external calibration curve for the analytes of interest. The overall purpose for the external calibration curve was to display acceptable linearity ($r^2 \geq 0.9801$) of the assigned calibration range. The recoveries of the analyte from the fortified samples were calculated by multi-point calibration.

Recovery results were computed for each sample. The equation used for quantification is presented in Appendix 2. A statistical treatment of the data includes the calculation of means, standard deviations (SD), RSDs as percentages (%), and the 95% confidence intervals. All statistics were calculated using Microsoft® Office Excel 2010.

### 4.1 Method Establishment

The ethephon transition from m/z 142.90 to 106.80, with the IS ($d_4$-ethephon) transition from m/z 146.90 to 111.00, both in negative mode, were used to quantitate ethephon. The ethephon transition from m/z 106.8 to 78.80, with the IS ($d_4$-ethephon) transition from m/z 146.90 to 111.00, both in negative mode, were used to confirm ethephon quantitation.

Prior to performing the ILV, the analyte retention times, instrument detection limits, and linearity of instrument responses to a range of analyte concentrations were determined, and the test system was verified as free of interferences at appropriate retention times.

### 4.5 Communication with Study Monitor

The Study Director notified the Study Monitor that Trial 1 was successful for ethephon but unsuccessful for 2-HEPA. After ruling out several potential issues with the method, it was decided that there was a significant carryover issue. The Study Director and Bayer Representative made several attempts to find the cause of this issue and to discover a solution, but neither party was successful.

The Bayer Representative directed CPS to terminate the experimental portion of the study and to write the final report based on the analysis of the parent compound. The Bayer Representative confirmed that CPS should include information about the failure of the metabolite but to focus the report on the success of the parent.

## 5.0 CONCLUSIONS

CPS successfully completed and independently validated the Bayer Method ET-001-S13-01 (see Appendix 5), entitled "An Analytical Method for the Determination of Residues of Ethephon and its Metabolite 2-HEPA in Soil and Sediment Using LC/MS/MS", for ethephon. Bayer Method ET-001-S13-01 was demonstrated to be suitable for the determination of ethephon in soil studied at both LOQ (5 ppb) and 10×LOQ (50 ppb) levels.

Bayer Method ET-001-S13-01 was not validated for the determination of 2-HEPA in soil, due to the instrumentation capability. The validation for 2-HEPA was terminated after two failed trials.

The method was performed as written with no major modifications.

It took one person approximately 4 hours to complete the extraction of one set of 13 samples (one reagent blank, two untreated control samples, and 10 fortified samples) for ethephon extraction. Time of LC-MS/MS analysis was approximately 6 hours. To complete one set, including extraction and analysis, took approximately 1 day.

**TABLE 2 HPLC SYSTEM OPERATING PARAMETERS FOR BAYER METHOD ET-001-S13-01 FOR ETHEPHON**

| | |
|---|---|
| HPLC System: | Agilent Model 1200® |
| Software: | Applied Biosystems, Analyst® 1.5.1 |
| Analytical Column: | Phenomenex® Aqua C18, 150 mm x 4.6 mm, 3 µm partical size |
| Mobile Phase: | (A): 0.1% formic acid in HPLC water<br>(B): Acetonitrile |
| Column Temperature: | 20°C |
| Injection Volume: | 25.0 µL |
| Draw Speed: | 100 µL/min |
| Eject Speed: | 200 µL/min |
| Run Time: | 8.0 minutes |
| Needle Wash: | Flush Port: 5 seconds using methanol/ water, 50:50, v/v |
| Gradient: | |

| Time (min) | A (%) | B (%) | Flow (µL/min) |
|---|---|---|---|
| 0.00 | 95.0 | 5.0 | 1000 |
| 3.00 | 95.0 | 5.0 | 1000 |
| 3.50 | 5.0 | 95.0 | 1000 |
| 4.50 | 5.0 | 95.0 | 1000 |
| 5.00 | 95.0 | 5.0 | 1000 |
| 8.00 | 95.0 | 5.0 | 1000 |

## TABLE 3 MS/MS OPERATING PARAMETERS FOR ETHEPHON

Tandem Mass Spectrometry System, Applied Biosystems™, API 4000™
Software: Applied Biosystems™, Analyst® 1.5.1

The following parameters were used for operation of the mass spectrometer:

| Parameter | Setting |
|---|---|
| Ion Source: | Turbo Spray |
| Scan Type: | MRM |
| Polarity: | Negative |
| Collision Gas (CAD): | 12 |
| Curtain Gas (CUR): | 30 |
| Ion Source Gas 1 (GS1): | 60 |
| Ion Source Gas 2 (GS2): | 70 |
| Ion Spray Voltage (IS): | -4500 |
| Temperature (TEM): | 600 |
| Interface Heater (IHE): | OFF |
| Declustering Potential (DP): | -51 |
| Entrance Potential (EP): | -10 |
| Collision Energy (CE): | -12 |
| Collision Cell Exit Potential(CXP): | -7 |
| Transitions Monitored: | Q1 (m/z) → Q3 (m/z) |
| Ethephon (Quantitation): | 142.9 → 106.8 |
| $d_4$-Ethephon (Quantitation): | 146.9 → 111.0 |
| Ethephon (Confirmation): | 106.8→ 78.8 |
| $d_4$-Ethephon (Confirmation): | 146.9 → 111.0 |

## APPENDIX 2 CALCULATIONS

For calculation of the concentrations, calibration curves were used. These curves were calculated automatically after each sequence run with the Applied Biosystems™, Analyst® software version 1.5.1 using linear regression. Further calculations were performed using the software Microsoft® Office Excel 2010.

The linear equation is expressed as:

$$y = Ax + B$$

where $y$ = Native peak area
$x$ = Concentration of the reference standard in ng/mL
$A$ = Calibration line slope
$B$ = Calibration line intercept

By means of the linear equation, the content of ethephon in Final Sample can be calculated as follows:

$$\text{Analyzed Final Sample Concentration (ng/mL)} = Ax + B$$

Theoretical Final Sample Concentration can be calculated as following:

$$\text{Theoretical Final Sample Concentration (ng/mL)} = \frac{C_s \times 1000 \times V_s}{W} \times DF$$

where
$C_s$ = Spiking solution concentration
(1.00 µg/mL and 10.0 µg/mL for LOQ and 10 × LOQ, respectively)
$V_s$ = Spiking solution volume (0.100 mL)
$W$ = Initial sample weight (20 ± 0.05 g)
$DF$ = Dilution Factor, which was calculated as following:

$$\text{Dilution Factor (DF)} = \frac{W}{V_E}$$

where $W$ = Initial sample weight (20 ± 0.05 g)
$V_E$ = Initial sample extraction solution volume (40 mL)

Calculate recoveries using the following equation:

$$\text{Recovery (\%)} = \frac{\text{Analyzed Final Sample Concentration}}{\text{Theoretical Final Sample Concentration}} \times 100$$

**Bayer Method ET-001-S13-01**

An Analytical Method for the Determination of Residues of Ethephon and its metabolite 2-HEPA in Soil and Sediment Using LC/MS/MS

## 1.0 SUMMARY

An analytical method was developed to determine the residues of Ethephon and its metabolite 2-HEPA in soil and sediment and is based on Bayer method 00899.[1]

Residues of ethephon are extracted from soil and sediment using water with phosphoric acid with microwave extraction. An isotopic internal standard is added to the sample and an aliquot is centrifuged. The supernatant is placed into a vial for analysis by LC/MS/MS with quantification based on a comparison of peak areas with those of known standards.

Residues of 2-HEPA are extracted from soil and sediment using water with formic acid with microwave extraction. An isotopic internal standard is added to the sample and an aliquot cleaned through MCX and MAX SPE cartridges. The SPE eluent is evaporated to dryness, taken up in water with formic acid and placed into a vial for analysis by LC/MS/MS with quantification based on a comparison of peak areas with those of known standards.

The method limit of quantitation (LOQ) in all sample matrices for ethephon and its metabolite 2-HEPA is 5 ng/g.

## 2.0 BACKGROUND

The analytical method presented in this report is designed to measure residues of ethephon and its metabolite 2-HEPA in soil and sediment using isotopically labeled internal standards and LC/MS/MS detection.

## 3.0 APPARATUS

(Functional equivalents may be substituted)

- Various general laboratory glassware and utensils
- MicroMan pipettors and tips
- Eppendorf Centrifuge 5810
- Milestone Ethos E Microwave Labstation, equipped with a Terminal 640 Touch Screen Controller and automatic temperature control with fiber optic sensor
- TurboVap
- Phenomenex Aqua C18, 150 mm X 4.6 mm, 3 µm particle size, (Part No: 00F-4311-E0)
- ABSciex API 4000 chromatograph/mass spectrometer (LC-MS/MS) equipped with electrospray ionization (ESI) interface, Shimadzu HPLC pumps and a CTC PAL autosampler, and Analyst 1.6.1 data collection software (ABSciex)

**4.0 REAGENTS AND CONSUMABLES**

(Functional equivalents may be substituted)

- Acetonitrile (Optima Grade, Fisher Part No. A996-4 )
- Formic Acid 99% (Acros, Part no. 14793-0010)
- Water (Optima Grade; Fisher Part No. W7-4)
- Methanol (optima Grade, Fisher Part No. A456-4)
- Phosphoric acid 85% (Acros Part no. 295700010)
- 0.1% formic acid in water. Add 1 mL formic acid to 1000 mL water. Mix well.
- 0.4% formic acid in water. Add 4 mL formic acid to 1000 mL water. Mix well.
- 0.7% phosphoric acid in water. Add 7 mL of phosphoric acid to 1000 mL water. Mix well.
- 2% formic acid in water:methanol (1:1, v:v). Add 2 mL of formic acid, 50 mL of methanol and 48 mL of water. Mix well.
- Fisherbrand 125mL 4oz glass jars (Part No. 02-911-455)
- Fisherbrand 2 mL microcentrifuge tube (Part No. 02-681-266)
- HPLC vials and caps (2-mL, National Scientific, Part Nos. C4011-5W and C4011-55)
- Disposable culture tubes, 15 mL (Kimble Chase Part No. 73790-15)
- Disposable stir bars, 1 x 5/16 (Fisher Part No. 1451394)
- Waters Oasis MAX, 6 mL, 500 mg extraction cartridge (Waters Part No. 186000865)
- Waters Oasis MCX, 6 mL, 500 mg extraction cartridge (Waters Part No. 186000776)

**5.0 PREPARATION OF STANDARD SOLUTIONS**

Ethephon and 2-HEPA analytical standards and the isotopic internal standards ethephon-$d_4$ and 2-HEPA-$d_4$ are needed. These standards may be obtained from Bayer CropScience, 2 T.W. Alexander Drive, Research Triangle Park, North Carolina, 27709. Additional details about these chemicals are given in Appendix 1.

The toxicities of these chemicals have not been precisely determined. Thus, each chemical must be treated as a potential health hazard. Exposure to these chemicals should be reduced to the lowest reasonable level.

NOTE: The following procedure is an example description of how these standard solutions may be prepared. Alternate or additional standards of appropriate weight and volume may be prepared as needed.

Class "A" volumetric glassware or calibrated pipets should be used in the preparation of all analytical standards. All standard stock solutions should be stored in a freezer when not in use and fortification and calibration standard solutions should be stored in a refrigerator when not in use. Solutions should be allowed to warm to room temperature prior to use. Corrections for standard purities should be applied when expressing standard concentrations.

**5.1 Primary Stock Standard Solution**

**5.1.1 Ethephon Primary Stock Standard Solution**

100 µg/mL solution of Ethephon
Standards used to prepare initial stock solutions should be weighed on an analytical balance capable of accurately weighing samples to ± 0.01 mg. Standards are typically provided in 9.0 to 12.0 mg aliquots. The standards are quantitatively transferred to a 100 mL volumetric flask using 0.7% phosphoric acid in water and diluted to volume.

**5.1.2 2-HEPA Primary Stock Standard Solution**

100 µg/mL solution of 2-HEPA
Standards used to prepare initial stock solutions should be weighed on an analytical balance capable of accurately weighing samples to ± 0.01 mg. Standards are typically provided in 9.0 to 12.0 mg aliquots. The standards are quantitatively transferred to a 100 mL volumetric flask using 0.4% formic acid in water and diluted to volume.

**5.2 Fortification Standard Solutions**

**5.2.1 Ethephon Fortification Standard Solutions**

10 µg/mL solution of Ethephon
Prepare a 10 µg/mL solution of ethephon by taking an appropriate volume (~10.0 mL) of the ethephon primary stock solution and diluting to 100 mL with 0.7% phosphoric acid in water.

1 µg/mL solution of Ethephon

Transfer 10 mL of the 10 µg/mL ethephon standard solution into a 100 mL volumetric flask. Dilute to volume with 0.7% phosphoric acid in water. Mix well.

**5.2.2 2-HEPA Fortification Standard Solutions**

10 µg/mL solution of 2-HEPA
Prepare a 10 µg/mL solution of 2-HEPA by taking an appropriate volume (~10.0 mL) of the 2-HEPA primary stock solution and diluting to 100 mL with 0.4% formic acid in water.

1 µg/mL solution of 2-HEPA

Transfer 10 mL of the 10 µg/mL 2-HEPA standard solution into a 100 mL volumetric flask. Dilute to volume with 0.4% formic acid in water. Mix well.

**5.3 Isotopic Internal Standard Solutions**

**5.3.1 Ethephon Internal Standard Solutions**

100 µg/mL solution of Ethephon-$d_4$
Standards used to prepare initial stock solutions should be weighed on an analytical balance capable of accurately weighing samples to ± 0.01 mg. Standards are typically provided in 2.0 to 5.0 mg aliquots. The standards are quantitatively transferred to a 50 mL volumetric flask using 0.7% phosphoric acid in water and diluted to volume.

10 µg/mL solution of Ethephon-$d_4$
Prepare a 10 µg/mL solution of ethephon-$d_4$ by taking an appropriate volume (~10.0 mL) of the 100 µg/mL ethephon-$d_4$ internal standard solution and diluting to 100 mL with 0.7% phosphoric acid in water.

1 µg/mL solution of Ethephon-$d_4$

Transfer 10 mL of the 10 µg/mL ethephon-$d_4$ internal standard solution into a 100 mL volumetric flask. Dilute to volume with 0.7% phosphoric acid in water. Mix well.

**5.3.2 2-HEPA Internal Standard Solutions**

100 µg/mL solution of 2-HEPA-$d_4$
Standards used to prepare initial stock solutions should be weighed on an analytical balance capable of accurately weighing samples to ± 0.01 mg. Standards are typically provided in 2.0 to 5.0 mg aliquots. The standards are quantitatively transferred to a 50 mL volumetric flask using 0.4% formic acid in water and diluted to volume.

10 µg/mL solution of 2-HEPA-$d_4$
Prepare a 10 µg/mL solution of 2-HEPA-$d_4$ by taking an appropriate volume (~10.0 mL) of the 100 µg/mL 2-HEPA-$d_4$ internal standard solution and diluting to 100 mL with 0.4% formic acid in water.

1 µg/mL solution of 2-HEPA-$d_4$

Transfer 10 mL of the 10 µg/mL 2-HEPA-$d_4$ internal standard solution into a 100 mL volumetric flask. Dilute to volume with 0.4% formic acid in water. Mix well.

**5.4 Calibration Standard Solutions**

**5.4.1 Ethephon Calibration Standard Solutions**

Prepare working calibration solutions consisting of 0.8, 2, 5, 10, 50 and 100 ng/mL of ethephon diluted to 50 mL with 0.7% phosphoric acid in water. Before bringing the calibration solutions to volume, add by pipet 0.5 mL of the 1 µg/mL ethephon-$d_4$ internal standard solution to each of the calibration solutions.

| Concentration of Standard Solution used for dilution (µg/mL) | Concentration of Internal Standard Solution used for dilution (µg /mL) | Aliquot Native mix Taken (mL) | Aliquot Internal Standard Taken (mL) | Dilution Volume (mL) | Concentration of Calibration Solution (ng/mL) | Concentration of Internal Standard (ng/mL) |
|---|---|---|---|---|---|---|
| 10 | 1 | 0.5 | 0.5 | 50 | 100 | 10 |
| 10 | 1 | 0.25 | 0.5 | 50 | 50 | 10 |
| 1 | 1 | 0.5 | 0.5 | 50 | 10 | 10 |
| 1 | 1 | 0.25 | 0.5 | 50 | 5 | 10 |
| 1 | 1 | 0.1 | 0.5 | 50 | 2 | 10 |
| 1 | 1 | 0.04 | 0.5 | 50 | 0.8 | 10 |

Further calibration solutions may be prepared as needed. Depending on the analytical range for the soil samples, at least six calibration standards are needed.

#### 5.4.2 2-HEPA Calibration Standard Solutions

Prepare working calibration solutions consisting of 0.8, 2, 5, 10, 50 and 100 ng/mL of 2-HEPA diluted to 50 mL with 0.4% formic acid in water. Before bringing the calibration solutions to volume, add by pipet 1.25 mL of the 1 µg/mL 2-HEPA-$d_4$ internal standard solution to each of the calibration solutions.

| Concentration of Standard Solution used for dilution (µg/mL) | Concentration of Internal Standard Solution used for dilution (µg /mL) | Aliquot Native mix Taken (mL) | Aliquot Internal Standard Taken (mL) | Dilution Volume (mL) | Concentration of Calibration Solution (ng/mL) | Concentration of Internal Standard (ng/mL) |
|---|---|---|---|---|---|---|
| 10 | 1 | 0.5 | 1.25 | 50 | 100 | 25 |
| 10 | 1 | 0.25 | 1.25 | 50 | 50 | 25 |
| 1 | 1 | 0.5 | 1.25 | 50 | 10 | 25 |
| 1 | 1 | 0.25 | 1.25 | 50 | 5 | 25 |
| 1 | 1 | 0.1 | 1.25 | 50 | 2 | 25 |
| 1 | 1 | 0.04 | 1.25 | 50 | 0.8 | 25 |

Further calibration solutions may be prepared as needed. Depending on the analytical range for the soil samples, at least six calibration standards are needed.

## 6.0 PROCEDURE

### 6.1 Sample extraction

#### 6.1.1 Sample extraction for Ethephon

Appendix 2A shows the analytical scheme for the extraction of ethephon in soil. The detailed stepwise procedure is as follows:

1. Weigh 20 ± 0.05 grams of soil/sediment into a 125 mL glass jar containing a magnetic stirbar.
2. Fortify the recovery samples at the desired fortification level with the appropriate standard solution.
3. Add 40 mL of 0.7% phosphoric acid in water to each sample.
4. Place jars with soil-solvent mixture into the microwave extractor.
5. Switch on the magnetic stirrer.
6. Extract for three minutes at 250 W.

7. Add 0.40 mL of the 1 µg/mL ethephon-$d_4$ internal standard solution. Mix well.
8. Transfer about 1.5 mL of the extract into a centrifuge tube. Centrifuge for 5 minutes at 10,000 rpm to remove fine particles of soil.
9. Transfer an aliquot to a vial for LC/MS/MS analysis.

**6.1.2 Sample extraction for 2-HEPA**

Appendix 2B shows the analytical scheme for the extraction of 2-HEPA in soil. The detailed stepwise procedure is as follows:

1. Weigh 20 ± 0.05 grams of soil/sediment into a 125 mL glass jar containing a magnetic stirbar.
2. Fortify the recovery samples at the desired fortification level with the appropriate standard solution.
3. Add 40 mL of 0.4% formic acid in water to each sample.
4. Place jars with soil-solvent mixture into the microwave extractor.
5. Switch on the magnetic stirrer.
6. Extract for three minutes at 250 W.
7. Add 0.100 mL of the 1 µg/mL 2-HEPA-$d_4$ internal standard solution. Mix well.
8. Centrifuge for 5 minutes at 2,000 rpm.
9. Apply a 5-mL aliquot to a preconditioned MCX cartridge with the effluent flowing into a clean culture tube. Cartridges are preconditioned with 10 mL of water. Do not use vacuum to pull the sample through the cartridge.
10. Rinse the MCX cartridge with 2-mL of methanol. Discard the MCX cartridge.
11. Apply the MCX effluent to a preconditioned MAX cartridge. Cartridges are preconditioned with 10 mL of water. Do not use vacuum to pull the sample through the cartridge.
12. Rinse the MAX cartridge with 2-mL of methanol. Discard the effluent.
13. Apply 5 mL of 2% formic acid in methanol:water (1:1) to the MAX cartridge and collect in a clean culture tube. Do not use vacuum to elute the cartridge.
14. Place the culture tube in a Turbovap set at 60°C and evaporate to dryness.

15. Add 0.5 mL of 0.4% formic acid in water to each sample tube, vortex and sonicate to dissolve all residues.

16. Transfer an aliquot to a vial for LC/MS/MS analysis.

**7.0 ANALYSIS BY LC-MS/MS**

**7.1 Analytical Procedure**

Step 1. Using the recommended procedures listed below, analyze an aliquot of each of the calibration standard solutions (if necessary, additional standard solutions may be added).

Step 2. Analyze an aliquot of each of the analytical samples.

**Note:** Up to 20 sample analyses can be made after the analysis of the standard solutions. In the case of over 20 samples, extra standard solutions could be added between sample analyses.

Step 3. Again, analyze an aliquot of each of the calibration standard solutions (and, if necessary, additional standard solutions).

**7.2 HPLC Conditions**

**Note:** The analyst should optimize chromatographic conditions to obtain satisfactory chromatography. The following recommended conditions were used on an ABSciex API 4000 instrument.

Mobile Phase A: Water containing 0.1% formic acid
Mobile Phase B: Acetonitrile

HPLC column: Phenomenex Aqua C18, 150 mm X 4.6 mm 3 µm particle size
Injection volume: 25-50 µL (Adjust for LC/MS/MS system being used)

| Time (min) | Ethephon Mobile Phase %B | 2-HEPA Mobile Phase %B | Flow rate (A &B) µL/min |
|---|---|---|---|
| 0.1 | 5 | 0 | 1000 |
| 3.0 | 5 | 0 | 1000 |
| 3.5 | 95 | 95 | 1000 |
| 4.5 | 95 | 95 | 1000 |
| 5.0 | 5 | 0 | 1000 |
| 8.0 | 5 | 0 | 1000 |

| Analyte | Approx Retention Time (min) |
|---|---|
| 2-HEPA | 1.8 |
| Ethephon | 2.7 |

### 7.3 Mass Spectrometer Conditions

Note: The analyst should optimize the mass spectrometer conditions to obtain satisfactory system response. The following conditions were used on a ABSciex API 4000 instrument.

Negative ion mode

| | |
|---|---|
| CUR: Curtain Gas | 30 |
| CAD: Collision Gas | 12 |
| GS1: Ion Source Gas 1 | 60 |
| GS2: Ion Source Gas 2 | 70 |
| TEM: Source Temp. | 600°C |
| IHE: Interface Heater | OFF |
| IS: Ion Transfer Voltage | -4500 |

Positive ion mode

| | |
|---|---|
| CUR: Curtain Gas | 30 |
| CAD: Collision Gas | 12 |
| GS1: Ion Source Gas 1 | 60 |
| GS2: Ion Source Gas 2 | 70 |
| TEM: Source Temp. | 600°C |
| IHE: Interface Heater | OFF |
| IS: Ion Transfer Voltage | 4500 |

### 7.4 Mass Spectrometer Data Collection

Note: The analyst should optimize the mass spectrometer data collection to obtain satisfactory system response. As the HPLC column ages, the retention times of the analytes will change. A standard solution should be analyzed before each set of samples to confirm the data collection parameters.

The daughter ions used in this method were chosen due to their optimum sensitivity on the ABSciex API 4000 instrument used for this study. The following recommended ion transitions and conditions were example conditions used on an ABSciex API 4000 instrument:

| Analyte Name | Polarity | Q1 Mass (amu) | Q3 Mass (amu) | Dwell (msec) | DP | EP | CE | CXP |
|---|---|---|---|---|---|---|---|---|
| Ethephon | - | 142.9 | 106.8 | 100 | -51 | -10 | -12 | -7 |
| Ethephon IS | - | 146.9 | 111.0 | 100 | -51 | -10 | -12 | -7 |
| Ethephon Confirmatory | - | 106.8 | 78.8 | 100 | -51 | -10 | -12 | -7 |
| 2-HEPA | - | 124.9 | 94.9 | 100 | -36 | -10 | -18 | -7 |
| 2-HEPA IS | - | 128.9 | 96.9 | 100 | -36 | -10 | -18 | -7 |
| 2-HEPA confirmatory | + | 127.1 | 99.0 | 100 | 56 | 10 | 27 | 6 |
| 2-HEPA IS Confirmatory | + | 131.1 | 99.0 | 100 | 56 | 10 | 27 | 6 |

## 8.0 CALCULATION OF RESULTS

The example calculation displayed below was used by the laboratory developing this method. Alternate calculation procedures appropriate to the reporting requirements may be substituted.

Residue concentrations were determined using calibration curves which were generated after each analysis using Analyst software (version 1.6) using linear regression with 1/x weighting.

The standards were fit to the linear equation:

$$Y = MX + B \text{ with } 1/x \text{ weighting.}$$

where: X is the concentration of the reference standard in ng/mL
M is the calibration line slope
B is the calibration line intercept
Y is the native peak area: isotopic peak area ratio

After regression coefficients were calculated, the residue in ng/g was determined using the following equation,

$$\text{Residue found (ng/g)} = \frac{(Y-B) \times D}{M}$$

Where Dilution Factor (D) = $\frac{\text{Initial volume } (V_1)}{\text{Initial sample wt. (W)}} \times \frac{\text{Final dilution volume } (V_3)}{\text{Aliquot taken } (V_2)}$

| | Ethephon | 2-HEPA |
|---|---|---|
| W= | 20 g | 20 g |
| $V_1$= | 40 mL | 40 mL |
| $V_2$= | 1 mL | 5 mL |
| $V_3$= | 1 mL | 0.5 mL |
| D= | 2 | 0.2 |

Analyst software was used to calculate the amount of ethephon and 2-HEPA in ng/g for each sample and the percent recovery for the spiked samples.

### 8.1 Fortification Experiments

Note: Fortification experiments may be performed as needed to monitor method efficiency and reproducibility, but are not required when analysis of samples is performed for tolerance enforcement. Fortification experiments are intended to be used for data collection methods or establishing and validating method efficiency.

With each sample set, analyze an untreated control sample and one or more fortified control samples. Calculate recoveries using the following equation:

$$\text{Recovery (\%)} = \frac{(R - S)}{T} \times 100$$

Where: R = ppb of target analyte found in fortified sample
S = ppb of target analyte found in control sample, real or apparent
T = theoretical ppb in fortified sample

Recoveries are determined by analyzing fortified control samples alone or in conjunction with a sample set. Samples may be fortified prior to extraction at the LOQ of 5 ng/g or other appropriate level with fortification solutions. Calculate the final residue for the control (S) and fortified control (R) samples.

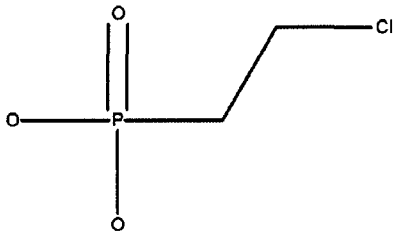
## 9.0 REFERENCES

1. Brumhard, B. Enforcement Method 00899 for the Determination of Residues of Ethephon in Soil by HPLC-MS/MS. 2004

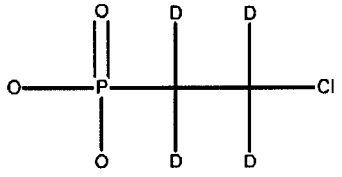
**Appendix 1 Test and Reference Substances**

The toxicities of these chemicals have not been precisely determined. Thus, each chemical must be treated as a potential health hazard. Exposure to these chemicals should be reduced to the lowest reasonable level.

Code Name: Ethephon
Molecular Formula: $C_2 H_6 Cl O_3 P$
Molecular Weight: 144.5 g/mol

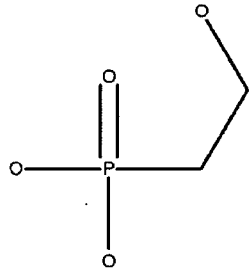



Code Name: Ethephon-$d_4$
Molecular Formula: $C_2 H_2 Cl D_4 O_3 P$
Molecular Weight: 148.5 g/mol

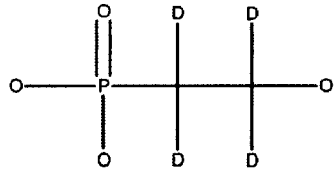


**Appendix 1 Test and Reference Substances (Cont'd)**

Code Name: 2-HEPA
Molecular Formula: $C_2 H_7 O_4 P$
Molecular Weight: 126.0 g/mol



Code Name: 2-HEPA-$d_4$
Molecular Formula: $C_2 H_3 D_4 O_4 P$
Molecular Weight: 130.1 g/mol



Bayer CropScience ET-001-S13-01

**Appendix 2A Extraction Scheme for Ethephon in Soil/Sediment Samples**

Weigh an aliquot of soil/sediment into a 125 mL glass jar
↓
Add ~40 mL of 0.7% phosphoric acid in water
↓
Microwave extraction. 250W for 3 minutes
↓
Add 0.4 mL of the 1 µg/mL ethephon internal standard solution
↓
Centrifuge an aliquot for 5 minutes at ~10,000 rpm
↓
Transfer an aliquot to a vial for LC/MS/MS analysis

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**Appendix 2B Extraction Scheme for 2-HEPA in Soil/Sediment Samples**

Weigh an aliquot of soil/sediment into a 125 mL glass jar
↓
Add ~40 mL of 0.4% formic acid in water
↓
Microwave extraction. 250W for 3 minutes
↓
Add 0.100 mL of the 1 µg/mL 2-HEPA internal standard solution
↓
Centrifuge for 5 minutes at ~2,000 rpm
↓
Apply a 5-mL aliquot of the supernatant to a pre-conditioned MCX cartridge
↓
Rinse MCX cartridge with methanol
↓
Apply the MCX cartridge effluent to a pre-conditioned MAX cartridge
↓
Rinse MAX cartridge with methanol
↓
Elute MAX cartridge with 5 mL of 2% formic acid in methanol/water (1:1, v/v).
↓
Evaporate sample to dryness
↓
Add 0.5 mL of 0.4% formic acid in water, vortex and sonicate
↓
Transfer an aliquot to a vial for LC/MS/MS analysis