3. CHEMICALS AND EQUIPMENT

3.1 Chemicals and Solvents

Acetonitrile, HPLC grade, Fisher Scientific
Hexane, HPLC grade, Fisher Scientific
Sodium Chloride, Reagent grade, Fisher Scientific

Water, Milli-Q, Millipore Corporation

3.2 Equipment

Analytical Evaporator, 12-port, "N-EVAP", Model 111, Organomation Assoc. Inc.
Balance, Analytical, Model 1602 MP, Sartorius
Balance, Top Loading, Model PL 1200, Mettler
Centrifuge, Beckman ®, Model TJ-6, Beckman Instruments
Centrifuge (bench), Centrific, Model 228, Fisher Scientific
Centrifuge tubes, 15 mL, polypropylene, FALCON ®, Becton Dickinson Labware
Centrifuge tubes, 50 mL, polypropylene, Fisherbrand ®, Fisher Scientific
Centrifuge tubes, 50 mL, polypropylene, Nalgene ®, Nalge Co.
Sonicator bath, Model FS60, Fisher Scientific
Tubes, 10 mL, graduated glass, Kolmer, Fisher Scientific
Vortex mixer, Model Genie 2 ®, Fisher Scientific
Customary analytical laboratory glassware and apparatus.

3.3 GC-MSD Instrumentation

The system consisted of an Agilent (Hewlett-Packard) gas chromatograph, Model 5890, with an autosampler-injector, Model 6890, a mass selective detector, Model 5972, linked to a Dell OptiPlex GX1 computer with NT Windows-based ChemStation, HP G-1701BA, and a Hewlett-Packard LaserJet 1200 printer.

Column:DB-1, (methylpolysiloxane), 30 m x 0.25 mm ID, x 0.25 μm film thickness, J & W Scientific.

4. TEST AND REFERENCE SUBSTANCES

4.1 Test Substance (Analytical Standard)

Chemical Abstracts Name: 2-[4-(1,1-methylethyl)phenoxy]cyclohexyl-2-propynyl Sulfite
IUPAC name: 2-(4-*tert*-butylphenoxy)cyclohexyl prop-2-ynyl sulfite
Common Name: Propargite
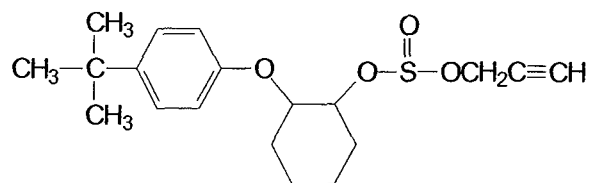CAS No: 2312-35-8
Crompton Corp. Analytical Standard Code: O-01
Lot No: AGD1827-049
Purity: 98.4%
Expiration Date: Jannuary 31, 2008
Molecular Weight: 350.47

Storage Condition: Freezer

4.2 Reference Substance for Propargite (Internal Standard)

Chemical Abstracts Name: 2-[3-(1,1-methylethyl) phenoxy]cyclohexyl-2-propynyl Sulfite
IUPAC Name: 2-(3-*tert*-butylphenoxy)cyclohexyl prop-2-ynyl sulfite
CAS No: Not available
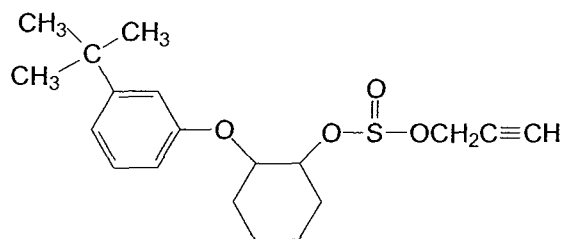Crompton Corp. Analytical Standard Code: O-63
Lot No: AGD1785-119
Purity: 99.5%
Expiration Date: May 31, 2006
Molecular Weight: 350.47
Storage Condition: Freezer

4.3 TBPC Metabolite (Analytical Standard)

Chemical Abstracts Name: 4-(1,1-dimethylethyl)phenoxy cyclohexane-1-ol
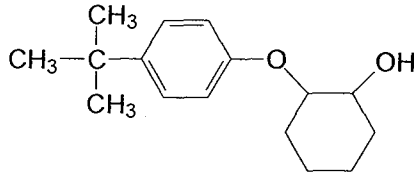IUPAC name: 4-(1,1-dimethylethyl)phenoxy cyclohexane-1-ol
Common Name: TBPC
CAS No: 1942-71-8
Crompton Corp.Analytical Standard Code: O-10
Lot No: AC1398-55
Purity: 99.6%

Expiration Date: July 31, 2006
Molecular Weight: 248.36
Storage Condition: Freezer

4.4 Reference Substance for TBPC (Internal Standard)

Chemical Abstracts Name: 3-(1,1-dimethylethyl)phenoxy cyclohexane-1-ol
IUPAC name: 3-(1,1-dimethylethyl)phenoxy cyclohexane-1-ol
Common Name: None
CAS No: None
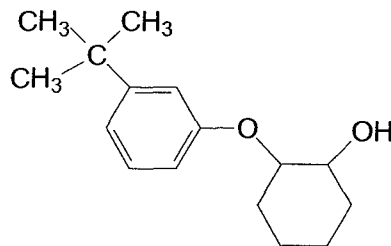Crompton Corp. Analytical Standard Code: O-66
Lot No: AGD-1785-110
Purity: 98.6%
Expiration Date: January 31, 2008
Molecular Weight: 248.36
Storage Conditions: Freezer

The Certificates of Analysis for the test and the reference substances are found in APPENDIX I.

5. TEST SYSTEM

The test system, sandy loam soil, from Stolpe, Germany was received from RCC Ltd., Itingen, Switzerland on 8/14/01 (Receiving Code RM-090) and stored in a refrigerator when not in use. The soil was characterized by RCC Ltd. (APPENDIX II).

6. PRINCIPLE OF THE METHOD

Propargite and TBPC were extracted from the soil with acetonitrile and centrifuged. The supernatant was decanted and concentrated to a small volume. The concentrated extract was diluted with 10% NaCl solution. The two analytes were extracted from the aqueous solution into hexane. Internal standards were added to the hexane extract. For samples fortified at the LOQ, the hexane extract containing the added internal standards was reduced and the volume adjusted with hexane, if needed, to 1 mL. Analysis was carried out using capillary gas chromatography (GC) with a mass spectrometer set in the selected ions monitoring mode (MSD). The ions m/z 150, 173 and 350 (molecular ion) were monitored for propargite and the ion m/z 173 for the propargite internal standard. The ions m/z 150, 233 and 248 (molecular ion) were monitored for TBPC and on m/z 150 for the TBPC internal standard.

7. PREPARATION OF 10% SODIUM CHLORIDE SOLUTION

Sodium chloride crystals, 25.07 g, were weighed into a 500 mL beaker. Water, 250 mL, (Milli-Q) was added to the beaker and the mixture was stirred until the crystals dissolved. The solution was stored in a plastic bottle at ambient temperature.

8. PREPARATION OF STANDARD SOLUTIONS

All stock and standard solutions were kept in a freezer when not in use.

8.1 Propargite Standard Stock Solution

Using an analytical balance, 54.8 mg of the standard was weighed into a 50 mL volumetric flask and the flask was filled to volume with acetone. The flask was sonicated. The concentration of the solution was adjusted for 100 % purity, using the equation:

$$1.08 \, mg \, / \, mL \, (100\%) = 54.8 \, mg \, / \, 50 \, mL \, x \, \frac{98.4}{100}$$

8.2 Propargite Standard Solution A

Stock solution, 0.94 mL (Section 8.1), was pipetted into a 100 mL volumetric flask and the flask was filled to volume with acetone. The concentration of the solution was 10.2 µg/mL. [(0.94 mL x 1.08 mg/mL)/100 mL x 1000 µg/mg].

8.3 Propargite Standard Solution B

Standard solution A, 5.0 mL (Section 8.2), was pipetted into a 50 mL volumetric flask and the flask was filled to volume with acetone. The concentration of the solution was 1.02 µg /mL [(5 mL x 10.1 µg/mL)/50 mL].

8.4 Internal Standard Stock Solution for Propargite

Using an analytical balance, 68.2 mg of the standard was weighed into a 50 mL volumetric flask. The flask was filled to volume with hexane and sonicated. The concentration of the solution for the internal standard was not adjusted for purity. The concentration of the solution was about 1.36 mg/mL (68.2 mg /50 mL).

8.5 Internal Standard Solution A for Propargite

Stock solution, 1.0 mL (Section 8.4), was pipetted into a 100 mL volumetric flask and the flask was filled to volume with hexane. The concentration of the solution was about 13.6 ug/mL [$13.6\ mg\,/\,mL\ x\dfrac{1.0\ mL}{100\ mL}x1000\ \mu g\,/\,mg$]

8.6 Internal Standard Working Solution B for Propargite

Standard solution, 5.0 mL (Section 8.5), was pipetted into a 50 mL volumetric flask and the flask was filled to volume with hexane. The concentration of the solution was about 1.36 µg/mL (13.6 µg/mL x 5 mL/50 mL).

8.7 TBPC Standard Stock Solution

Using an analytical balance, 51.9 mg of the standard was weighed into a 50 mL volumetric flask and the flask was filled to volume with acetone. The flask was sonicated. The concentration of the solution was adjusted for 100 % purity, using the equation:

$$1.03\,mg\,/\,mL\left(100\%\right)=51.9\,mg\,/\,50\,mL\,x\frac{99.6}{100}$$

8.8 TBPC Standard Solution A

Stock solution, 0.97 mL (Section 8.7), was pipetted into a 100 mL volumetric flask and the flask was filled to volume with acetone. The concentration of the solution was 10.0 µg/mL. [(0.97 mL x 1.03 mg/mL)/100 mL x 1000 µg/mg].

8.9 TBPC Standard Solution B

Standard spiking solution A, 5.0 mL (Section 8.8), was pipetted into a 50 mL volumetric flask and the flask was filled to volume with acetone. The concentration of the solution was 1.00 µg /mL [(5 mL x 10.0 µg/mL)/50 mL].

8.10 Internal Standard Stock Solution for TBPC

Using an analytical balance, 52.3 mg of the standard was weighed into a 50 mL volumetric flask. The flask was filled to volume with hexane and sonicated. The concentration of the solution for the internal standard was not adjusted for purity. The concentration of the solution was about 1.05 mg/mL (52.3 mg /50 mL).

8.11 Internal Standard Solution A for TBPC

Stock solution, 1.0 mL (Section 8.10), was pipetted into a 100 mL volumetric flask and the flask was filled to volume with hexane. The concentration of the solution was about 10.5 ug/mL [$1.05 \; mg/mL \; x \dfrac{1.0 \, mL}{100 \, mL} \, x 1000 \, \mu g / mg$]

8.12 Internal Standard Solution B for TBPC

Standard solution, 5.0 mL (Section 8.11), was pipetted into a 50 mL volumetric flask and the flask was filled to volume with hexane. The concentration of the solution was 1.05 µg/mL (10.5 µg/mL x 5 mL/50 mL).

8.13 Preparation of the Calibration Standards

Using syringes, the Propargite Internal Standard Solution A (O-63), 200 uL and TBPC Internal Standard Solution A (O-66), 100 uL were added to 10 mL volumetric flasks. Then Propargite Standard Solution A, and TBPC Standard Solution A, were added to the same 10 mL volumetric flasks, as described in the following Table. The flasks were filled to the mark with hexane.

Table. Dilutions for the Calibration Plot

Propargite µL	TBPC, µL	ISTD (O-63) µL	ISTD (O-66) µL	Propargite/TBPC µg/mL
50	50	200	100	0.05
70	70	200	100	0.07
100	100	200	100	0.10
150	150	200	100	0.15
200	200	200	100	0.20

Note: Calibration standards in the above range were employed for LOQ and 10 x LOQ quantitation.

The stock and standard solutions were prepared fresh before the start of the definitive study and were found to be stable, as shown by the GC-MSD response, over the course of the definitive study, which lasted for 20 days.

9. SAMPLE FORTIFICATION, EXTRACTION AND ANALYSIS

The validation was carried out as described in the amended analytical method AC –6018 (APPENDIX IV, page 65). The procedure was amended to include metabolite TBPC.

9.1 Fortification of the Soil Samples

The soil sample (approximately 10 g) was weighed into a Nalgene ® centrifuge tube and fortified with propargite and TBPC standards in acetone solution.

a. LOQ: 100 uL of the Propargite and TBPC Standard Solution B, was added to the sample.

b. 10 x LOQ: 100 uL of the Propargite and TBPC Standard Solution A, was added to the sample.

9.2 Extraction and Clean Up Procedure

Acetonitrile (25 mL) was added to the centrifuge tube and the tube was vortexed, sonicated and centrifuged. The supernatant was decanted into a clean (new) Fisherbrand ® centrifuge tube. The acetonitrile extraction was repeated. The combined extracts were reduced in volume to about 5 mL, under a stream of nitrogen in a water bath set at about 50 °C. The acetonitrile extract was

transferred to a 15 mL clean (new) FALCON ® centrifuge tube, using ACN rinse, 0.5 mL , of the 50 mL tube. The extract was further reduced in volume to about 1 mL, under a stream of nitrogen in a water bath set at about 50 °C. About 5 mL of 10% sodium chloride solution was added to the concentrated extract followed by 5.0 mL of hexane. The tube was vortexed and centrifuged. The hexane phase was transferred into a 15 mL clean (new) FALCON ® centrifuge tube. The aqueous phase was extracted with another 5.0 mL of hexane. The two internal standards were added to the combined hexane extracts and the final volume was adjusted with hexane to 10 mL. For samples fortified at LOQ, the extract was concentrated and adjusted with hexane, if needed, to 1.0 mL prior to analysis.

Note: To avoid potential contamination, the Fisherbrand ® and FALCON ® centrifuge tube were not reused.

9.3 Parameters for GC Operation

Carrier gas: He
Column head pressure: 9-10 psi
Injector temperature: 210 ° C
Injection volume: 2 µL (splitless)

Oven temperature program:

Hold 1 min at 60 ° C
Ramp 10 ° C/ min to 290 ° C
Hold 2 min at 295 ° C
Run time: 26.5 minutes
Retention time: a) Propargite ISTD, approximately 21.6 min; Propargite STD, Aproximately 22.2 min. b) TBPC ISTD approximately 17.1 min; TBPC STD approximately 17.7 min.

9.4 Parameters for MSD Operation

Transfer line: 300 ° C
EI ionization current (+eV): 35
Repeller (+eV): 25
Autotune range (+ meV): 1400-1500
Electron multiplier setting (+meV): 400-500 above autotune
TBPC Scan:
Detector ON at:16.0 minutes
Detector OFF at:18.5 minutes
Propargite Scan:

Detector ON at: 21.00 minutes
Detector OFF at: 22.50 minutes
Resolution: ± 0.1 amu
Dwell time (ms): 30
Cycles/sec: 7.14
<u>Ions to monitor (m/z)</u>: a) TBPC; m/z 150,150, 150, 233, 233, 233 and 248, 248, 248 (molecular ion) and m/z 150, 150, 150 for TBPC ISTD. b) Propargite; m/z 150, 150, 150, 173, 173, 173 and 350, 350, 350 (molecular ion). and m/z 173, 173, 173 for the propargite internal standard.

Note: To increase sensitivity, exact masses were used in triplicate, e.g. 173, 173, 173, rather than the usual ± 0.1 amu range per ion, for example, 172.90, 173.00, 171.10,

9.5 Sample Set

A sample set consisted of three controls soil samples and five fortified control soil samples. The quantitation was done using a five-level calibration plot bracketing the concentration of the analyte in the samples. Hexane blanks were employed between the injections of the standards and the samples.

10. METHODS OF CALCULATION

The calculations shown below were employed for propargite. The calculations for TBPC are the same, except that quantitation was done using the ratio of ions m/z 248 $_{STD}$/150 $_{ISTD}$. The confirmatory ions for TBPC were m/z 150 and 233.

10.1 Regression Analysis

The ratio of peak areas of the standard to that of the internal standard for the two ions employed for quantitation, (m/z 350 $_{STD}$/173 $_{ISTD}$), is the dependent variable and the concentration of the standard solution, expressed as µg/mL, is the independent variable. Using the calibration plot, a linear regression equation was generated to determine the intercept, the slope and the linearity of detector response, as expressed by correlation coefficient (R).

The linear regression was generated, using Microsoft Excel software, Version 9.0.0.5519.

$$Ratio_{STD} \ (STD/ISTD) = Intercept + Slope \ x \ (\mu g \ /mL)_{STD.}$$

10.2 Calculation of the Concentration (µg/mL) of Propargite in Hexane Extract

Using peak areas ratio of the m/z 350 ion in the sample or fortified control to that of internal standard, m/z 350 $_{Spl}$/173 $_{ISTD}$ (Ratio $_{Spl}$), in the hexane extract, the concentration of propargite is determined, using the following equation:

$$Propargite, \mu g / mL = \frac{(Ratio_{Spl} - Intercept)}{Slope}$$

If an interference peak is found in the control samples, then a corrected peak area value is determined using the following formula:

$$PeakArea_{Sample} \ (corrected) = PeakArea_{Sample} - PeakArea *_{control}$$

* A mean value for the two (or more) controls within a sequence. Under EU guidelines, the peak area of the interference should be 30% or less of that of the sample peak for this formula to be valid.

No interference was observed with the quantifying ions for propargite or TBPC.

Example SPK-4 (AppendixIII, Page 61)

Linear Regression Constants: Intercept = -0.0731
         Slope = 2.6612
         Ratio $_{Spl}$ = 0.19289797

$$\frac{[0.192897971 - (-0.0731)]}{2.6612} = 0.09996 \, \mu g / mL \, (propargite)$$

10.3 Calculation of the Amount (µg) of Propargite Found in Hexane Extract

Multiply the propargite concentration (µg/mL) determined in Section 10.2 by the volume of the hexane extract, expressed in mL:

$$\mu g / mL \, x \, Extract \, Volume \, (mL) = \mu g \, (propargite)$$

Example of Calculation (SPK-4)

$$0.09996 \, \mu g / mL \, x \, 1 \, mL = 0.09996 \, \mu g \, (propargite)$$

10.4 Calculation of the Amount (mg/kg) of Propargite Found in the Sample

Divide the amount (µg) of propargite determined in Section 10.3 by the sample weight, expressed in grams (g):

$$\frac{ug\ propargite}{Sample\ Weight\ (g)} = mg\ /\ kg\ (propargite)$$

Example of Calculation (SPK-4)

$$\frac{0.09996\ \mu g}{10\ g} = 0.01\ mg\ /\ kg\ (propargite)$$

10.5 Calculation of % Recovery of Propargite from the Fortified Control

Calculate % recovery by dividing the value (µg) determined in Section 10.3, by the amount of propargite added to the sample times 100%:

$$\frac{\mu g\ of\ propargite\ (Found)}{\mu g\ of\ propargite\ (Added)}\ X\ 100 = \%Recovery$$

Example of Calculation (SPK-4)
The amount of propargite found in the sample in Section 10.3 was 0.09996 µg and the amount added to the sample was 0.10 µg. The % recovery is:

$$\frac{0.09996\ \mu g}{0.10\ \mu g}\ x\ 100 = 100\%$$

The **95% Confidence Interval** for mean recovery are calculated using the expression:

$$\pm\ \frac{t\ x\ SD}{\sqrt{n}}$$

where **t** is the student critical value for 95 % confidence interval for (n-1) replicates and **SD** is the standard deviation for **n** replicates.

Example of Calculation (Samples fortified at LOQ, APPENDIX III, Page 61).

n = 5
SD = ± 9.78
Student t value (n-1) = 2.776*
$(n)^{1/2}$ = 2.236

The 95 % Confidence Interval is:

$$\frac{(2.776 \, x \, 9.78)}{2.236} = \pm 12.1\%$$

* Quality Assurance of Chemical Measurements, J. K. Taylor, APPENDIX C, page 267, Lewis Publishers, Inc. (1987).

10.6 Determination of the Identity of Propargite.

The identity of propargite is determined by dividing peak area of confirmatory ions m/z 173 and 150 by the peak area of the quantifying ion, m/z 350. The mean values must be within 20 % (USA) or 70% to 130% (EU) of the mean value of the same ratios calculated for the standards in the standard calibration plot.

Example of calculation (SPK-4, APPENDIX III, page 61)

Calculation of the ratio for each of the samples (or standards) is as follows:

m/z	SPK-4, Peak Area	Ratio, m/z173/350	Ratio, m/z 150/350
350	799244		
173	3532253	4.42	
150	1427633		1.79

The mean ratio is then calculated for the samples and the standards in the set.

	m/z 173/350	m/z 150/350
Samples Mean Ratios (n = 5)	4.57	1.93
Standards Mean Ratios (n = 6)	4.99	2.00

The mean values of the ratio for the samples and the standards varied by less than 10%.

$[(4.99-4.57)/4.99] \times 100 = 8.42\%$
$[(2.00-1.93)/2.00] \times 100 = 3.50\%$