MP-DICH-MA
PAGE 1 OF 11
DATE: 10/16/87
REPLACES: Original
SECTION: 6012

ANALYTE: Dichlobenil and 2,6-Dichlorobenzamide

AREA OF APPLICABILITY: Hazleton Laboratories America, Inc.
Chemical and BioMedical Division

SCOPE:

This method is applicable to the determination of dichlobenil and 2,6-dichlorobenzamide in soil samples.

PRINCIPLE:

The soil is extracted with hexane: acetone (50:50) and ammonium chloride solution followed by filtration. Water and salt are added to the solution and the hexane layer is saved. The aqueous layer is then extracted twice with hexane and combined with the original hexane layer. This solution contains dichlobenil. The aqueous layer is then extracted three times with ethyl acetate. This combined solution contains 2,6-dichlorobenzamide. The dichlobenil solution is oxidized. Following a GPC clean up step or alumina column clean-up step, the dichlobenil samples are concentrated by roto-evaporation, iso-octane is added and the sample is concentrated once again, then transferred to a 10 mL volumetric and diluted to mark. These samples are then analyzed by GC analysis with electron capture detection and 10% carbowax 20 M column. Following a GPC clean-up, or alumina column clean-up, i.e., the 2,6-dichlorobenzamide samples are roto-evaporated to dryness, then quantitatively transferred to a 10 mL volumetric flask with ethyl acetate and diluted to mark. These samples are then analyzed by GC analysis using electron capture detection off a 3% OV-225 column.

SENSITIVITY:

The sensitivity of this method is 0.01 mg/kg for both test compounds.

SAFETY PRECAUTIONS:

1. Avoid breathing fumes of hexane, methylene chloride, cyclohexane, iso-octane, ethyl acetate, and petroleum ether.

2. Observe all standard laboratory safety procedures as outlined in the Hazleton Laboratories America, Inc. Safety Training Manual.

QUALITY ASSURANCE:

Analysis sets of approximately 10 field samples will include one reagent blank, one duplicate field sample chosen at random, and an amended, previously-analyzed control.

APPARATUS:

1. 500 mL Erlenmeyer flasks with a 24/40 joint and stoppers.

2. Shaker, wrist-action.

3. Suction flasks, 500 mL.

4. Buchner funnels of appropriate size.

5. Fiberglass or paper filters of appropriate size.

6. Separatory funnel, 500 mL.

7. Funnels, powder.

8. Volumetric flasks, assorted sizes

9. Glass wool, solvent rinsed.

10. Roto-evaporation equipment.

11. GPC Auto-Prep, Model 1001, Analytical Biochemistry Laboratories, Columbia, Mo. equipped with a 600 x 25 mm column packed with 62 g Bio Beads S-X3. Operate according to proper SOP.

12. Glass chromatographic column, 30 cm x 9 mm, equipped with 100 ml reservoir, stopcock, and sintered glass frit.

13. GC equipment and conditions.

Chromatographic Conditions for Dichlobenil

Instruments: Hewlett-Packard Model 5710
Column: 6 ft x 4 mm i.d. packed with 10% Carbowax 20M on 100/120 mesh Chromosorb WHP
Detector: Ni^{63}, electron capture
Carrier gas: Argon:methane (95:5) at 86 mL/minute

Temperatures:
Column: 200°C
Injector: 200°C
Detector: 250°C
Chart speed: 2 mm/minute
Attenuation: varies
Injection volume: 5 uL

Chromatographic Conditions for 2,6-Dichlorobenzamide

Instruments: Hewlett-Packard Model 5710
Column: 3 ft x 2 mm i.d. packed with 3% OV-225 on 80/100 mesh Chromosorb WHP
Detector: Ni^{63}, electron capture
Carrier gas: Argon:methane (95:5) at 38 mL/minute
Temperatures:
Column: 200°C
Injector: 250°C
Detector: 350°C
Chart speed: 0.25 in./minute
Attenuation: varies
Injection volume: 4 uL

GC conditions may be modified as necessary to obtain satisfactory separation and peak shape.

REAGENTS:

1. Ammonium Chloride solution 0.20%.
(Weigh out 4.0g of NH_4Cl and place in a 2 L Vol. flask and dilute to mark with Milli-Q water. An equivalent recipe ratio can be used to make a smaller or greater volume of this reagent.

2. Hexane, PR grade, glass distilled.

3. Petroleum ether, PR grade, glass distilled.

4. Ethanol, U.S.P., dehydrated, 200 proof.

5. Ethyl Acetate, PR grade, glass distilled.

6. Acetone, PR grade, glass distilled.

7. Methylene Chloride, PR grade, glass distilled.

8. Hexane/Acetone 50:50 solution. (Measure 2 L of each solvent and pour into 4 L bottle. Mix by inverting bottle several times.)

9. Milli-Q Purified deionized water.

10. Saturated sodium chloride (NaCl) solution. Add granular NaCl to a flask containing Milli-Q water and dissolve the NaCl. Continue additions of NaCl until some NaCl remains undissolved. The solution is now saturated. The volume of water may vary depending upon assay needs.

11. Sodium Bicarbonate solution 0.1ON. (Add 8.4 g of solid $NaHCO_3$ to a one liter volumetric flask and dilute to mark with Milli-Q water.)

12. Saturated Potassium Permanganate in 0.1N sodium Bicarbonate. Add granular $KMnO_4$ (Potassium Permanganate) to an appropriate volume of 0.1N $NaHCO_3$ solution until no further additions of $KMnO_4$ will dissolve. The solution is now saturated.

13. Sodium meta-bisulfite, AR Grade.

14. Iso-octane, PR grade, glass distilled.

15. Cyclohexane, PR grade, glass distilled.

16. GPC Solvent 50:50 cyclohexane: methylene chloride. Prepare by adding 2 liters of cyclohexane to 2 liters methylene chloride in a 4-liter solvent bottle. Mix by inverting several times.

17. Bio Beads S-X3, 200 - 400 mesh, Bio Rad Laboratories, Richmond, CA.

18. Acetone: Petroleum Ether 2:98 solution (measure 2 mL of acetone into each 98 mL of Petroleum Ether) prepare fresh daily.

19. Acetone: Petroleum Ether 10:90 solution (measure 400 mL of acetone and 3,600 ml of Petroleum Ether into a 4 liter bottle. Mix by inverting several times.)

20. Ethanol: Petroleum Ether 15:85 solution. (Measure 600 mL of Ethanol and 3,400 mL of Petroleum Ether into a 4 L bottle. Mix by inverting several times).

21. Alumina (Al_2O_3, Merck-Darmstadt). Heat in a 120°C oven for 24 hours. Allow to cool in a desiccator, pour into a 1 L Erlenmeyer flask, add 2% w/w of water using a pipet. Shake on a wrist action shaker for at least 4 hours. Keep top tightly closed.

STANDARDS:

The analytical standards for the parent compound and its primary metabolite were provided by Duphar, B. V., Weesp, Holland.

Parent Material

Chemical name:	2,6-Dichlorobenzonitrile
Common name:	Dichlobenil
Chemical abstracts registry number:	1194-65-6
Structure:	

Lot number:	ARS82I01N
Purity:	Greater than 99.5%
Empirical formula:	$C_7H_3Cl_2N$
Molecular weight:	172.02
Physical form:	White crystals

Primary Metabolite

Chemical name:	2,6-Dichlorobenzamide
Common name:	2,6-Dichlorobenzamide
Chemical abstracts registry number:	2008-58-4
Structure:	

Lot number:	ARS81C25N
Purity:	Greater than 99.0%
Empirical formula:	$C_7H_5Cl_2NO$
Molecular weight:	190.0
Physical form:	White powder

STANDARD PRESCRIPTION:

Weigh 0.100 g each compound and transfer to separate 100 mL volumetric flask using acetane. Dilute to mark and mix by inverting several times. Resulting concentration 1000 µg/mL.

Working standards are prepared by diluting in appropriate solvent (iso-octane for Dichlobenil, ethyl acetate for 2,6-Dichlorobenzamide, or acetone for spiking solutions.) Examples of standard preparation follow:

Initial Concentration (µg/mL)	Aliquot (mL)	Final Volume (mL)	Resulting Concentration (µg/mL)
1000	10	100	100
1000	1	100	10.0
100	1	100	1.00
1.00	10	100	0.100
1.00	8	100	0.080
1.00	5	100	0.050
1.00	3	100	0.030
1.00	1	100	0.010
1.00	0.5	100	0.005

Additional standard levels may be prepared as required.

PROCEDURE:

1. Weigh a 25-g sample into a 500-mL Erlenmeyer flask equipped with a 24/40 joint. If sample is a recovery spike at this point.

2. Add 17 mL of 0.20% ammonium chloride and 150 mL of hexane:acetone (50:50). Note: If sample is a field spike or storage stability sample, rinse container with these solutions.

3. Stopper flask and shake on a wrist-action shaker for at least 2 hours.

4. Using suction, filter sample through a Buchner funnel, lined with a filter, into a 500-mL flask. Rinse original Erlenmeyer and filter with 30 mL acetone/hexane (50:50).

5. Transfer extract to a 500 mL separatory funnel, add 10 mL saturated NaCl solution and 300 mL Milli-Q purified water.

6. Shake separatory funnel for about 1 minute, venting as necessary to relieve pressure. Allow layers to separate.

7. Drain lower aqueous layer back into the 500-mL suction flask, then pass the organic layer through anhydrous sodium sulfate supported by a powder funnel and glass wool into a 500-mL roundbottom flask.

8. Transfer aqueous layer back into the separatory funnel, repeating extraction twice with 100 mL hexane, combining each extract. This solution contains dichlobenil.

9. Repeat extraction three times with 100 mL ethyl acetate and collect this portion in a separate 500-mL roundbottom flask. This solution contains 2,6-dichlorobenzamide.

10. The hexane layer is evaporated to about 50 mL on a rotary film evaporator at less than 40°C.

11. Transfer the 50 mL hexane extract to the previously used 500 mL separatory funnel, add 15 mL of a saturated $KMnO_4$ solution. Shake for 1 minute. Solution will turn a purple color.

12. Add 150 mL of Milli-Q water and mix. Add solid $Na_2S_2O_5$ until the excess $KMnO_4$ has been removed. The solution will go clear.

13. Draw off the aqueous layer. Wash the hexane layer with 2 - 25 mL portions of water. Filter hexane layer through a powder funnel with glass wool plug and anhydrous sodium sulfate (Na_2SO_4) into a 250 mL roundbottom flask. Rinse the funnel and the Na_2SO_4 with hexane into the round bottom flask.

14. GPC clean up. Note: GPC (14a-14d) or alumina (15a-b) clean up may be used.

    a. Both the oxidized extract and ethyl acetate extracts are evaporated to about 5 mL on a rotary film evaporator at less than 40°C. Note: do not allow the oxidized extract to go to dryness. Add about 20 mL of 50% cyclohexane-methylene chloride to each and repeat evaporation to about 5 mL. Transfer to separate 25 mL volumetric flasks using 50% cyclohexane-methylene chloride.

b. Five-milliliter sample extracts are cleaned up using a GPC Autoprep, Model 1001, Analytical Bio-Chemistry Laboratories, Inc., Columbia, Missouri, with the following conditions:

Column: 600 mm x 25 mm i.d. packed with 62 g Bio-Beads SX-3, Bio-Rad Laboratories, Richmond, California
Flow rate: 4.7 mL/minute
Solvent: 50% cyclohexane-methylene chloride
Dump time: 33 minutes
Collect time: 40 minutes
Rinse time: 5 minutes

Collect eluant in 500 mL roundbottom flasks.

c. Roto evap the oxidized samples to approximately 5 mL. Note: Do not allow the oxidized samples to go to dryness. Add 25 mL of iso-octane and reduce to 5 mL with roto-evaporization. Transfer these samples to a 10 mL volumetric flask and dilute to mark.

d. Roto evap the 2,6-dichlorobenzamide samples to dryness and transfer to a 10 mL volumetric flask with ethyl acetate.

15. Alumina Column Clean-up

a. Dichlobenil

1. Evaporate the oxidized extracts to about 10 mL on a rotary film evaporator at less than 40° C. Transfer with hexane to a 25 mL volumetric flask. Note: do not allow the extracts to go to dryness.

2. Fill the chromatographic columns with the deactivated alumina to a depth of 6 cm. Wash each column with 25 mL of Petroleum ether.

3. Pipette 5 mL of the hexane portion onto the column and rinse inside of column with 5 mL Petroleum Ether. Chromatagraph, if necessary, under nitrogen or air pressure, and discard the effluent. After sample has reached the level of the alumina wash with 25 mL Petroleum Ether and discard the effluent. Elute with 25 mL of 2:98 acetone: Petroleum Ether, into a 125 mL Erlenmeyer flask with a 24/40 ground glass joint.

4. Add approximately 10 mL of Iso-octane to the elute and evaporate to approximately 5 mL on a rotary flash evaporator. Transfer to a 10 mL volumetric flask and dilute to mark. This solution is ready for gas chromatographic quantitation.

b. 2,6-dichlorobenzamide

1. Evaporate the ethyl acetate extracts to dryness on a rotary film evaporator at less than 40°C. Transfer with 10:90 acetone: Petroleum ether to a 25 mL volumetric flask.

2. Fill the chromatographic columns with the deactivated alumina to a depth of 6 cm. Wash each column with 25 mL of Petroleum Ether.

3. Pipette 5 mL of the extract in acetone:Petroleum Ether (10:90) solution onto the column and rinse the inside of the column with 25 mL of 10:90 acetone:Petroleum Ether. Chromatograph, if necessary, under nitrogen or air pressure and discard the effluent. After the sample has reached the level of the alumina, wash with 15 mL of 10:90 acetone:Petroleum Ether and discard the effluent. Elute with 30 mL of 15:85 ethanol:Petroleum Ether into a 125 mL Erlenmeyer flask with 24/40 ground glass joint.

4. Evaporate the elute on a rotary film evaporator to dryness and absorb residue in 10:00 mL ethyl acetate (pipette 10:00 mL ethyl acetate into flask and pour contents into disposable culture tube, and cap with a teflon line cap.) This solution is ready for gas chromatographic quantitation.

16. Analyze the dichlobenil samples by GC using the 10% Carbowax 20 M column and electron capture detection. Analyze the 2,6-Dichlorobenzamide samples by GC using the 3% OV-225 column and electron capture detection. Each GC run will include appropriate standards and samples.

17. Percent moisture will be assessed by weighing approximately 25 grams of the wet soil, baking it to remove all moisture then reweighing dried sample.

CALCULATE:

To calculate mg/kg, a standard curve was generated using linear regression analysis of standard response versus concentration. Sample responses were subsequently compared to the curve and concentrations were generated.

$$mg/g = U \times C \times D$$

Where U = concentration determined from curve

C = ratio of final sample volume to original sample volume

D = dilution factor

To calculate percent recovery, the following formula was used:

$$\text{percent recovery} = \frac{\text{mg/kg found}}{\text{mg/kg added}} \times 100$$

For percent moisture and percent solids, the following formula is used:

$$\text{percent moisture} = \frac{(WW + C) - (DW + C)}{(WW + C) - C} \times 100$$

Where: WW = wet weight

DW = dry weight

C = container weight

percent solids = 100-percent moisture

To correct for percent moisture, the following was used:

$$\text{mg/kg corrected} = \frac{\text{mg/kg uncorrected}}{\frac{\text{percent recovery}}{100} \times \frac{\text{percent solids}}{100}}$$

APPENDIX E

Deviations from Dichlobenil and 2,6-Dichlorobenzamide Determination Method and Protocol for HLA Study No. 6012-181

Deviations to the Method for
HLA Study No. 6012-181

- In the cleanup step we used alumina columns instead of GPC.
- The chart speed recorder was at 0.5 mm/minute instead of 2 mm/minute for the 2,6-dichlorobenzamide.
- For better separation, the GC column packing for the 2,6-dichloro-benzamide was 1.5% SP-225G/1.95% SP-2401 instead of 3% OV-225 on 80/100 mesh chromosorb WHP.
- The flow rate was varied instead of using one setting, (38 to 50 mL/minute).
- About 20 g of soil samples were used for moisture instead of 25 g.
- To avoid homogenity problems in sampling the field spiked soil sample that was analyzed for dichlobenil and 2,6-dichlorobenzamide, the total weight was used (100 g) for analysis instead of 25 g as in the method.
- Occassionally we ran into GC sensitivity change problems that caused us to use two different standard curves to calculate samples within the set. See raw data tables.
- GC condition labels for 2,6-dichlorobenzamide on the chromatograms stated:

  Column temperature = 195°C, the correct temperature used was 200°C
  Detector temperature = 350°C, the correct temperature used was 300°C

Deviations to the Analytical Protocol
for HLA Study No. 6012-181

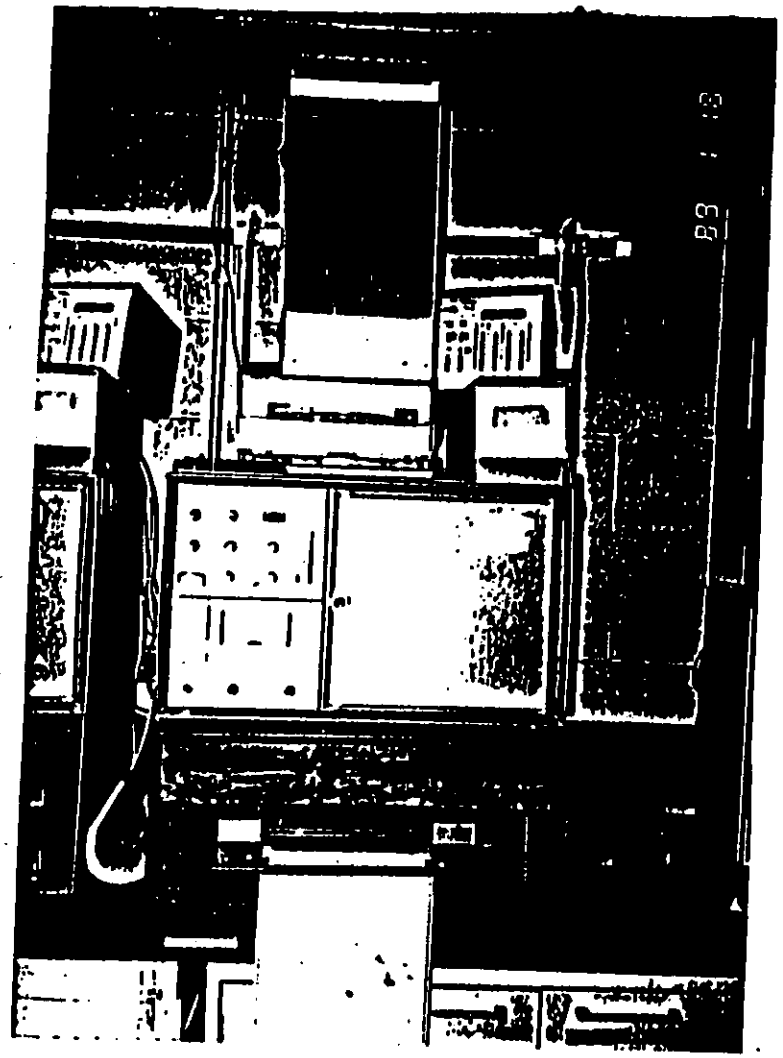
- Dr. Milton Ganyard's address (Principal Field Investigator) was changed during the study without establishing an amendment to the protocol. The address changed from 4006 Barrett Drive to 4401 Bland Road, Raleigh, North Carolina.
- Set No. 7 was analyzed without a duplicate sample for the 2,6-dichlorobenzamide due to sample loss during analysis.
- On Page 6 of this report the Sponsor name was

changed to:	instead of:
Duphar B.V. Crop Protection Division P.O. Box 4 1243 ZG's-Graveland, Holland	Duphar B.V. Analytical Department C. J. Van Houtenlaan 36 138 CP Weesp, Holland

- The method validation table on Page 46 has a late addition of 0.01 ppm (limit of detection), which was requested by the Client. This is a deviation from the protocol, which stated that the lowest level of validation is 0.05 ppm.

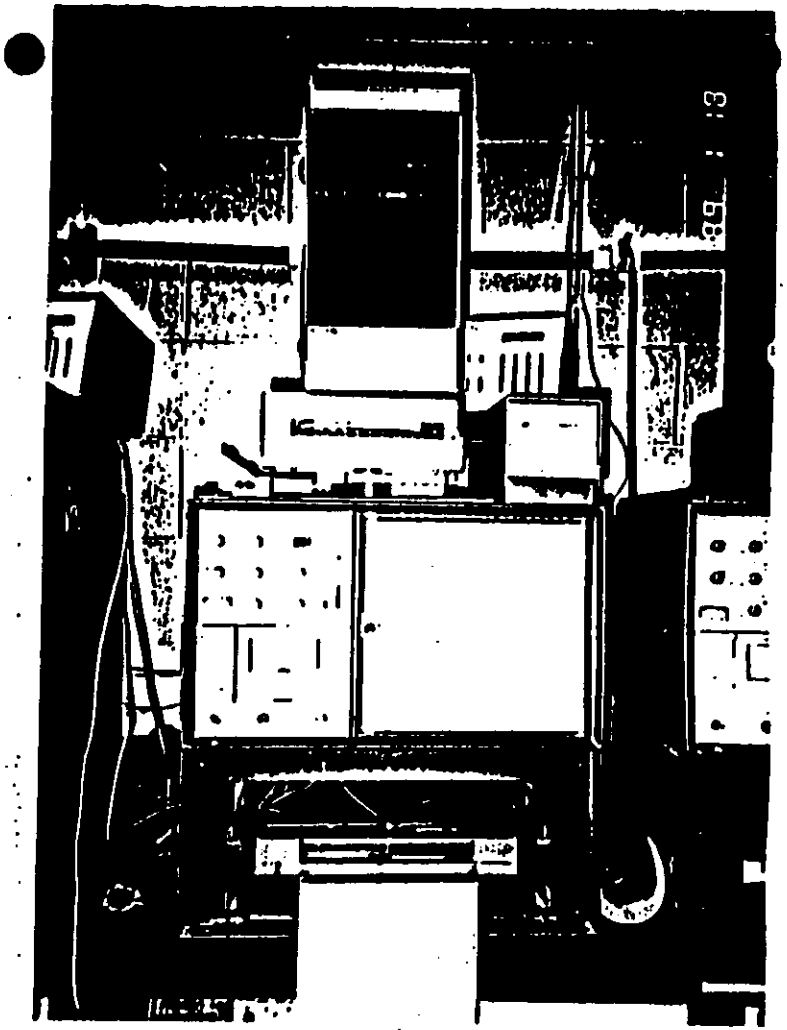
APPENDIX F

Instrument and Operating Conditions for Dichlobenil and 2,6-Dichlorobenzamide in Soil



HP-5710-ECD NO. 0006000050
COLUMN: GLASS; 6 ft x 4 mm
PACKING: 10% CARBOWAX 20M
TEMP. OF: INJ.=200°C; COL.=200°C; DET.=250°C
CARRIER GAS: P-5 Ar/METH; RATE: 86 ml/min.
RECORDER 876979 ; 1mv FS; SPEED: 0.5cm/min
ATTENUATION: varies WORKSHEET NO. NA

Was for methane



HP-5710-ECD # 0008000034
COLUMN: GLASS; 6' X 4mm
PACKING: 1.5% SP-2250 /1.95% SP-2401
TEMP. OF :INJ.=250°C; COL.=195°C; DET.=350°C
CARRIER GAS: P-5 Ar/METH; RATE: 40 ml/min
RECORDER 876124 ; 1mv FS; SPEED: 0.5cm/min
ATTENUATION: varies WORKSHEET # NA

Was for methane/ammonia

APPENDIX G

Calculations

Calculations

To calculate mg/kg of Dichlobenil or 2,6-Dichlorobenzamide

$$mg/kg = \frac{Y \times V \times D}{W}$$

Y = Concentration (µg/mL) obtained from the standard curve
V = Volume (25 mL)
D = Dilution factor
W = Sample weight in grams

A hand calculator was used to obtain the linear regression.

$$Y = mX + b$$

Y = Sample concentration (µg/mL)
m = Slope
X = Sample response (mm)
b = Y-intercept
R = Correlation of coefficient

Example: 25 g of soil sample was weighed for analysis into 25 mL
A 5 mL sample was taken and diluted to 10 mL

slope = m = 5.23026×10^{-4} n = Y-intercept = -0.588521×10^{-3}
response = X = 145 mm

$$(\mu g/mL) = Y = mx + b$$
$$= 5.23026 \times 10^{-4} \times 145 +(-) 0.588521 \times 10^{-3}$$
$$= 0.0752\ \mu g/mL$$

$$mg/kg\ (ppm) = \frac{0.0752 \times 25 \times 2}{25} = \frac{Y \times V \times D}{W}$$
$$= 0.15\ ppm$$

To Calculate Percent Recovery

$$\text{Percent recovery} = 100 \times \frac{\text{ppm found}}{\text{ppm added}}$$

Example: The control soil (25 g) was weighed and fortified with 1 mL of 10-µg/mL standard (0.4 ppm).

If ppm found = 0.389 ppm

$$\text{Percent recovery} = \frac{0.389}{0.40} \times 100 = 97.2\%$$

Calculations (Continued)

To Calculate Percent Moisture

$$\text{Percent Moisture} = \frac{(WW + C) - (DW + C)}{(WW + C) - C} \times 100$$

WW = Wet weight
DW = Dry weight
C = Dish weight

Example: Dish weight = 1.57 g, sample wet weight = 20.0 g
sample dry weight = 14.85

$$\text{Percent moisture} = \frac{21.57 - 16.42}{21.57 - 1.57} \times 100 = \frac{5.15}{20} \times 100 = 25.8\%$$

Data Correction

This equation was used for data correction of average percent recovery and percent moisture.

$$\text{mg/kg corrected} = \frac{\text{mg/kg uncorrected}}{\frac{\% \text{ Recovery}}{100} \times \frac{\% \text{ Solids}}{100}}$$

Example: The ppm (mg/kg) of dichlobenil for one of the soil samples was equal to 1.5 ppm. Percent moisture for this sample was 21.0%. The average percent recovery was equal to 90%.

$$\text{Corrected mg/kg dichlobenil} = \frac{1.50}{\frac{90}{100} \times \frac{79}{100}} = 2.11 \text{ (mg/kg)}$$

Information About Software for Plotting Graphs

The data used to plot the following graphs (Figures 1 through 5) were computer generated from the original raw data using Symphony version 2.0 software (License No. 16070005), Lotus Development Corporation (Lotus), 55 Cambridge Parkway, Cambridge, MA 02142. Symphony rounded data to two places to the right of the decimal and rounded up at the number 5 regardless of the next number to the right.

Example: 9.365 becomes 9.37

Samples designated "NA" (not assayed) were considered zero in performing calculations. Samples reported as "less than 0.01 were considered 0.01 for calculating and plotting purposes.

Standard deviation data was calculated as "sample" standard deviation [illegible]

The Symphony generated data was then entered as plotting points in version 3.11 of Sigma-Plot software program (License No. 107812), Jandel Scientific, 65 Koch Rd., Corte Madera, CA 94925