ABSTRACT

The following report presents the results of an independent laboratory validation for the determination of dichlobenil and 2,6-dichlorobenzamide in water by gas chromatography using a mass specific detector. The method validated was developed by Uniroyal Chemical Company, Inc. and is titled, "Analytical Method for Determining Dichlobenil and its Metabolite 2,6-Dichlorobenzamide in Water (Analytical Method No. AC-7005, Uniroyal study number 99055).

The analytical method consists of passing a 1 L water sample through a divinylbenzene (DVB) Speedisk™ solid phase extraction disk under vacuum. The dichlobenil and its metabolite, 2,6-dichlorobenzamide (BAM), which are retained on the disk, are eluted with methylene chloride and gently concentrated under nitrogen with 1-heptanol as a keeper. For quantitation purposes, an internal standard, 2,4,6-trichlorobenzonitrile, is added to reference standards and the concentrated samples. Analysis is by GC/MS in the selected ion monitoring (SIM) mode. The limit of quantitation (LOQ) for the method is 0.10 ug/L (ppb).

The ILV trial consisted of one reagent blank, duplicate controls, five control samples fortified at 0.10 ug/L (1X LOQ) and five controls fortified at 1.0 ug/L (10X LOQ). The acceptable range for the individual recoveries was 70-120%. The method was validated in the first trial.

FIGURE 1. FLOW SCHEME FOR THE DETERMINATION OF DICHLOBENIL AND 2,6-DICHLOROBENZAMIDE IN WATER

Clean a 50 mm Bakerbond DVB Speedisk™ using 15 mL each acetone, n-propanol, and methanol, drying between solvents.

↓

Condition Speedisk™ using 10 mL methanol followed by 25 mL NANOpure™ water (15 mL then 10 mL without allowing disk to dry between solvents.

↓

Extract 1.0 L water sample at ~80 mL/min under vacuum.

↓

Elute analytes using 15 mL methylene chloride
(3 X 5 mL each)

↓

Add 160 uL of 1-heptanol as a keeper.

↓

Concentrate sample under very gentle nitrogen stream at room temperature to ~1400 uL.

↓

Add 100 uL of 2,4,6-trichlorobenzonitrile internal quantitation standard solution.

↓

GC/MS analysis using selected ion monitoring, HP-5MS, 30 m X 0.25 mm X 0.25 um
70°C for 1 min, 10°C/min to 270°C for 1 min monitor m/z 171, 173, 175, 189, 205, and 207

↓

Quantitation from average of two 5-point calibration curves of analyte/internal quantitation standard ratio bracketing the range of analyte concentrations observed in the samples.

INTRODUCTION

This report presents the results of an independent laboratory validation (ILV) for the determination of dichlobenil and 2,6-dichlorobenzamide in water by gas chromatography with a mass specific detector. The information which follows includes a description of the experimental design, the test substances/analytical reference standards, the test system, the analytical methodology, and calculations. Results can be found in Tables 1and 2 of this report and example chromatograms can be found in Appendix B.

EXPERIMENTAL DESIGN

TEST SUBSTANCES / ANALYTICAL REFERENCE STANDARDS

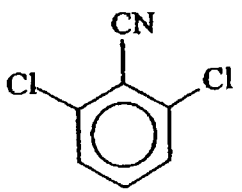
Source for analytical standards: Uniroyal Chemical Company, Inc., Middlebury, CT
Descriptions:

Name	Percent Purity	Physical State	CAS#	Lot#	Expiration Date
dichlobenil	100%	Solid	1194-65-6	ARS-09108-BA	03/31/2006
2,6-dichlorobenzamide*	100%	Solid	2008-58-4	ARS-81C25N	03/31/2006
2,4,6-trichlorobenzonitrile	97%	Solid	6575-05-9	03417 (Syn.#)	11/26/2004

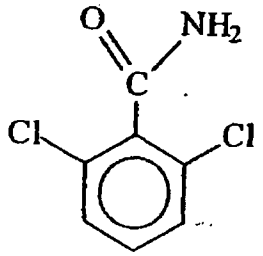
dichlobenil = 2,6-dichlorobenzonitrile
2,4,6-trichlorobenzonitrile = internal standard. Lancaster Synthesis supplied internal standard to Uniroyal Chemical Company.
*2,6-dichlorobenzamide may be referred to as "BAM" in the raw data associated with this report.

The chemical structures are as follows:

Dichlobenil



2,6-dichlorobenzamide



The purities of the analytical standards used in this study were verified by Uniroyal Chemical Company, Middlebury, CT. .The neat analytical reference standards and analytical reference standard solutions were stored frozen, in amber vials when not in use. The preparation of the standard solutions was recorded in a standard logbook; a list of the stored stock analytical standard solutions prepared during this study is presented in Table 4.

TEST SYSTEM AND TEST SYSTEM CHARACTERIZATION

The laboratory validation was performed using pond water from Arcata, California. A 5 gallon carboy, four 4 L amber bottles and a 1 L brown Nalgene bottle were filled with pond water using a 1 quart dipper. Each container was identified with a unique NCL number and stored refrigerated, except when it was being sub-sampled for analysis. The pond water in the 1 L brown Nalgene bottle was shipped, in a cooler containing blue ice, by overnight courier, to Agvise Laboratories, Inc., Northwood, ND.

Agvise Laboratories, Inc determined the chemical properties of the pond water. Total organic carbon was 3.8 ppm, total suspended solids were 20 ppm, and total dissolved solids were 72 ppm. These and other data are shown in Appendix E.

LIMITS OF QUANTITATION (LOQ)

The LOQ for dichlobenil and 2,6-dichlorobenzamide was 0.10 ug/L (ppb).

SAMPLE ANALYSIS DESIGN

The validation set consisted of the following samples:

1 reagent blank	
2 controls	
5 recoveries	dichlobenil at 0.10 ug/L (ppb)
	2,6-dichlorobenzamide at 0.10 ug/L (ppb)
5 recoveries	dichlobenil at 1.0 ug/L (ppb)
	2,6-dichlorobenzamide at 1.0 ug/L (ppb)

METHODOLOGY

The analytical method described in Uniroyal Chemical Company method, "Analytical Method for Determining Dichlobenil and its Metabolite 2,6-Dichlorobenzamide in Water" (Analytical Method No. AC-7005, Uniroyal study number 99055) was followed with only minor modifications, as described in Appendix A.

Standard solution preparation:

For the calculations involved in the preparation of the following standard solutions, see the calculations section of this report.

The solvent used in the preparation of fortification standards was methanol, and the solvent used in the preparation of calibration standards was dichloromethane.

- Preparation of 1,000 ng/μL (μg/mL) mixed analyte (i.e., dichlobenil and 2,6-dichlorobenzamide) fortification and calibration stock standard solutions:

Approximately 10-11 mg of each neat analytical standard was weighed out and, taking into account the percent purity, was brought to the appropriate volume with methanol to yield a 1,000 ng/μL standard solution for each analyte.

- Preparation of a 10 ng/μL (μg/mL) fortification and calibration mixed analyte stock standard solution:

A 100 uL aliquot of each of the two 1,000 ng/μL stock standard solutions was transferred into the same 10 mL volumetric flask and brought to volume with methanol.

- Preparation of a 1 ng/μL (μg/mL) fortification and calibration mixed analyte stock standard solution:

A 1.0 mL aliquot of the 10 ng/μL mixed analyte stock standard solution was transferred to a 10 mL volumetric flask and brought to volume with methanol.

- Preparation of linear and calibration mixed analyte working standard solutions:

Working standards were prepared at 0.050, 0.075, 0.10, 0.125, 0.150 ug/L for the analysis of the 0.1 ug/L fortification samples and at 0.50, 0.75, 1.0, 1.25 and 1.50 for the 1.0 ug/L fortification samples.

-Preparation of stock internal standard solution which is added to each standard and sample:

The internal standard was prepared at 1.0 ug/L for addition to the 0.10 ug/L fortifications and to the 0.05, 0.075, 0.10, 0.125, and 0.150 ug/L working standard solutions. The internal standard was prepared at 10.0 ug/L for addition to the 1.0 ug/L fortification samples and the 0.50, 0.75, 1.0, 1.25 and 1.50 ug/L working standard solutions.

- Preparation of 1,000 ng/μL (μg/mL) internal standard stock standard solution:

Approximately 10-11 mg of the neat internal standard was weighed out and, taking into account the percent purity, was brought to the appropriate volume with methanol to yield a 1,000 ng/μL standard solution.

- Preparation of a 10 ng/μL (μg/mL) internal standard solution:

A 100 uL aliquot of the 1,000 ng/μL stock standard solution was transferred into a 10 mL volumetric flask and then brought to volume with methanol.

- Preparation of a 1 ng/μL (μg/mL) internal standard stock standard solution:

A 1.0 mL aliquot of the 10 ng/μL internal standard stock standard solution was transferred to a 10 mL volumetric flask and then brought to volume with methanol.

Sample preparation:

Sample preparation was not necessary.

Fortification:

A 1.0 liter aliquot of pond water was measured using a graduated cylinder and poured into a 1 L glass bottle. Using a 100 uL syringe, 100 uL of a 1 ng/uL mixed analyte stock standard was added to prepare the 0.10 ug/L fortifications and 100 uL of a 10 ng/uL mixed analyte stock standard was added to prepare the 1.0 ug/L fortifications. The fortified samples were mixed by capping the bottle and shaking.

Extraction:

A DVB Speedisk ™ with reservoir was placed onto a vacuum manifold attached to a small Baker Box. A beaker serving as a waste receptacle was placed under the disk. Fifteen (15) mL of acetone were passed through the disk under vacuum and pulled through the disk until the disk was dry. The disk was rinsed again with 15 mL n-propanol followed by 15 mL of methanol. The disk was conditioned with 10 mL methanol by allowing the methanol to flow through slowly by gravity. When 3-5 mL of methanol remained on the disk, 15 mL of NANOpure™ water was added. The water was allowed to flow through by gravity. When 3-5 mL of water remained on the disk, an additional 10 mL of water were added and allowed to flow through by gravity until about 5 mL of water remained. Without letting the disk go dry, the 1 L water sample was added and the vacuum adjusted to achieve a flow rate of about 80 mL/minute. The entire water sample was processed without letting the disk go dry.

After the sample had passed through the disk, the reservoir was removed and the disk remained under full vacuum for about 3 minutes in order to completely dry the disk. The vacuum was then released.

A 15 mL KD vial with a 2 mL tip was placed under the port and a total of 15 mL methylene chloride was added to the disk in three aliquots of about 5 mL each. The methylene chloride was eluted by applying a vacuum.

Concentration:

One hundred and sixty (160) uL of 1-heptanol was added as a keeper solvent to the top of each sample. The samples were gently swirled to mix the keeper with the sample. The samples were concentrated to 1300-1400 uL each under a very gentle stream of nitrogen in a room temperature water bath. Internal standard, 100 uL of 10.0 ug/mL 2,4,6-trichlorobenzonitrile working standard solution, was added and the samples were vortexed to obtain a homogeneous mixture. The extracts were transferred with a Pasteur pipette to an autosampler vial for

GC/MS analysis.

Analysis:

Identification and quantitation of dichlobenil and 2,6-dichlorobenzamide were by gas chromatography with a mass specific detector in the selected ion monitoring mode. Identification was made by comparing GC retention times and mass spectra (ratio of ions monitored) of the peaks in sample extracts with those of dichlobenil and 2,6-dichlorobenzamide analytical reference standards. Quantitation was done by comparing signal response (area counts) for the analyte quantitation ion against signal response (area counts for the internal standard quantitation ion. The ratio of analyte area counts to internal standard area counts was calculated for each sample and this ratio was compared to the analogous ratio on a five-point standard calibration curve.

INSTRUMENTATION

Analysis of dichlobenil and 2,6-dichlorobenzamide was performed using a Hewlett Packard (HP) 6890 gas chromatograph, a HP 7683 autosampler, and a HP 5973 mass specific detector. A HP- 5MS, 30 meter, 0.25 mm I.D., 0.25 mm film thickness column was used for chromatography. The injection volume was 1.5 uL. See Appendix D for chromatography conditions.

CALIBRATION PROCEDURES

A fixed injection volume of 1.5 μL was used for quantitation purposes and was the same for both samples and standards. Five calibration standards were injected before and after each set of validation samples. An internal standard, 2,4,6-trichlorobenzonitrile, was added at a concentration of 0.1 ug/L to the 0.1 ug/L fortification standards and samples and 1.0 ug/L to the 1.0 ug/L standards and samples. The low fortifications, 0.10 ug/L, were bracketed by two sets of mixed analyte standards at the concentrations of 0.05, 0.075,.1.0, 1.25, and 1.5 ug/L. The high level fortification samples, 1.0 ug/L, were bracketed by two sets of mixed analyte standards at the concentrations of 0.50, 0.75, 1.0, 1.25 and 1.50 ug/L. The data from the two bracketing sets of standards were combined to generate a single calibration plot used to quantify each fortification level. A calibration curve for each analyte was generated by plotting the ratios of analyte to internal standard as a function of analyte concentration. The average responses were calculated by HP Chemstation software and confirmed by Microsoft Excel.

Linearity of detector response was determined for each analyte by calculating a coefficient of determination (R^2) for the calibration curve used to quantify sample values. The coefficient of determination was required to be $\geq$ 0.99 for the calibration to be considered linear. The low and high level fortification calibration standard curves for both analytes had coefficients of determination (R^2) of 1.000.

QUALITY CONTROL

Quality control for the validation consisted of analyzing one reagent blank, two control samples, five calibration standards at the beginning of the run and five calibration standards at the end of the run. The analytical order was: calibration standards for 0.10 ug/L fortification samples (from low to high), rinse, reagent blank, control samples, 0.10 ug/L fortification recovery samples, calibration standards for 0.10 ug/L fortification samples (from low to high), rinse, calibration standards for 1.0 ug/L fortification samples, rinse, 1.0 ug/L fortification samples, and calibration standards for 1.0 ug/L fortification samples. The runs were considered acceptable if the coefficient of determination (R^2) was $\geq$ 0.99 for the calibration curves generated by averaging the beginning and ending five standard sets.

CALCULATIONS

Threshold area counts

The term "NAC" (no area counts), found on the raw data sheets and in Table 2, means that there were no peaks available to report at or above the area reject threshold.

Calculations for method

- Calculation of the amount of solvent needed to bring a weighed amount of standard to the required concentration:

$$\text{Volume of solvent} = \frac{\text{Weight of standard (adjusted for percent purity)}}{\text{Desired concentration}}$$

Example - Preparation of the 1,000 ug/mL dichlobenil stock standard solution:

Weight dichlobenil = 0.0114 g
Percent purity = 100%
Weight of dichlobenil =
(0.0114 g) (1,000,000 ug/g) = 11,400 ug

$$\text{Volume of solvent needed} = \frac{11{,}400 \text{ ug}}{1{,}000 \text{ ug/mL}} = 11.40 \text{ mL}$$

- Calculations in the preparation of the recovery samples:

The 0.10 ug/L (ppb), 1X LOQ, recovery samples were prepared by fortifying 1 L of pond water with 100 uL of 1.0 ng/uL of a mixed analyte fortification stock standard solution:

(100 μL) (1.0 ng/μL) /1.0 L = 100 ng/L = 0.10 ug/L (ppb)

The in-solution concentration of the final extract from a 1X LOQ fortification sample at 100% recovery with a final volume of 1 mL is:

(100 μL) (1.0 ng/μL) (1/1 mL) = 100 ng/mL = 0.10 ug/mL

The 1.0 μg/L (ppb), 10X LOQ, recovery samples were prepared by fortifying a 1 L of pond water with 100 μL of a 10.0 ng/μL mixed analyte fortification stock standard solution.

(100 μL) (10.0 ng/μL) (1/1.0 L) = 1000 ng/L = 1.0 ug/L

- Preparation of a 0.10 μg/L (ppb) mixed analyte calibration working standard solution:

A 100 uL aliquot of the 1.0 ng/μL mixed analyte standard was brought to 1.0 mL with dichloromethane. The concentration with respect to the sample size of 1 L with a final extract volume of 1 mL is:

(100 μL) (1.0 ng/μL) (1 mL/1 mL) (1/1 L) = 100 ng/L = 0.10 ug/L

The in-solution concentration of this standard is:

(100 μL) (1.0 ng/μL) (1/1 mL) = 100 ng/mL = 0.10 μg/mL.

This "agrees" with the in-solution concentration of a final extract from a 0.10 μg/L fortification at 100% recovery.

Calibration and linear working standards at other concentrations were prepared in a similar manner.

Calculations used to create the calibration curve used for quantitation

Ratios of analyte/internal standard peak areas of the standards (dichlobenil or 2,6-dichlorobenzamide) were the dependent variables and the nominal concentrations of each analyte in the standard solutions (ug/L) were the independent variables. These values were used to generate a calibration curve with a linear regression equation to determine the intercept, slope and linearity of the responses for each analyte.

$Ratio_{standard}$ = slope X nominal standard concentration (ug/L) + intercept

Calculation of the amount of analyte in a sample extract

The amount of analyte (diclobenil or 2,6-dichlorobenzamide) in the extract was calculated by using the following equation:

$$\text{Analyte (ug/L)} = \frac{(\text{ratio}_{\text{sample}} - \text{intercept})}{\text{slope}}$$

Calculation of percent recovery in fortified samples

$$\text{Method recovery (\%)} = \frac{\text{µg/L calculated sample residue}}{\text{µg/L fortification concentration}} \times 100$$

Example calculations:

The following are examples of these calculations for dichlobenil in the sample described below. The chromatogram for this sample can be found in Figure B11 of Appendix B.

Sample Description:	Recovery #5 at 0.10 µg/L
NCL ID No.:	0104587-01F
Analyte:	Dichlobenil
Extraction date:	05/21/01
Analysis date:	05/23/01
Dilution factor:	1.0
Line No.:	19

Linear regression analysis equation for the mean of the beginning and ending five point calibration curve resulted in the following values:

Slope = 1.46656
Intercept = -0.02668

$$\text{Ratio}_{\text{sample}} = \frac{\text{86619 area counts of sample}}{\text{70829 area counts of the internal standard}} = 1.2229$$

$$\text{Dichlobenil (ug/L)} = \frac{[1.2229 - (-0.02668)]}{14.6656 \text{ L/ug}} = 0.0852 \text{ ug/L}$$

$$\text{Method recovery (\%)} = \frac{0.0852 \text{ ug/L}}{0.10 \text{ µg/L fortification concentration}} \times 100 = 85.2\%$$

2. DESCRIPTION OF ANALYTICAL METHOD

a. Method report number: Uniroyal Chemical Company, Inc. AC-7005, Uniroyal study number 99055.

b. Title of method: "Analytical Method for Determining Dichlobenil and its Metabolite 2,6-Dichlorobenzamide in Water"

c. Scope of method: The Uniroyal Chemical Company, Inc. analytical method described is intended for the determination of residues of diclobenil and 2,6-dichlorobenzamide in water from a lower limit of quantitation of 0.010 ug/L (ppb).

d. Minor modifications to the method:

III. E. GC/MS INSTRUMENTATION

The column used was a HP-5MS, 30 meter, 0.25 mm I.D., 0.25 um film thickness

F. GC/MS ANALYSIS PROCEDURE

F-3 GC/MS Operating Conditions

The following conditions were modified from those specified in F-3 of the method:

Column head pressure:	1.7 PSI
Injection volume:	1.5 uL
Oven temperature program:	
Initial oven temperature:	60^0 C

Ramp: (°C/min.)	Final temp.: (°C)	Final Time (min.)
10.00	220	0
40.0	280	2.0

Run Time:	20.5 min.
Equilibration Time:	1.0 min.

3. FULL DESCRIPTION OF ANALYTICAL INSTRUMENTATION USED

A Hewlett Packard 6890 gas chromatograph equipped with a HP 5973 mass specific detector, a HP 7683 autosampler, and a HP-5MS, 30 meter, 0.25 mm I.D., 0.25 um

film thickness were used for the quantitation of dichlobenil and 2,6-dichlorobenzamide. The injection volume was 1.5 μL. Calibration and calculation of analyte concentrations were performed using HP Chemstation software.

4. DESCRIPTION OF ANY PROBLEMS ENCOUNTERED IN CONFIRMING THIS METHOD

Step D6: The methylene chloride would not elute by gravity. A substantial vacuum was needed. A small amount of water (a few drops) came through with the methylene chloride. The KD vials (used to collect the eluate) were therefore centrifuged at 1000 RPM for 3 minutes and the water removed with a Pasteur pipette. The analyst was concerned that the water might interfere with evaporation.

During the analysis a retention time shift occurred for dichlobenil and the internal standard, 2,4,6-trichlorobenzonitrile. The retention time of dichlobenil was 10.95 minutes in the reference standards and 11.19 minutes in the samples. The retention time of the internal standard was 12.20 minutes in the reference standards and 12.31 minutes in the samples. The retention time for 2,6-dichlorobenzamide, which does not contain a cyano group, did not a shift. The reason for these retention time shifts was not investigated.

5. IDENTIFICATION OF CRITICAL STEPS

None

6. APPROPRIATENESS OF THE PREDETERMINED LIMITS OF QUANTITATION

Limit of quantitation is reasonable.

7. PERSON HOURS REQUIRED TO COMPLETE ONE SET OF 13 SAMPLES

8 hours

8. NUMBER OF CALENDAR DAYS REQUIRED FOR ONE SUBSET OF 7 SAMPLES

1 day. Complete analysis could be done in 24 hours. Extraction could go faster if equipment was set up to extract more samples at the same time.

APPENDIX D

CHROMATOGRAPHY CONTIDIONS

Instrument:

GC:	HP 6890 gas chromatograph
MSD:	HP 5973 mass specific detector
Autosampler:	HP 7683
Column:	HP-5MS, 30 meter, 0.25 mm I.D., 0.25 um film thickness
Column head pressure:	1.7 PSI
Injector temperature:	250°C
Injection volume:	1.5 uL

Oven temperature program

Initial temperature:	60°C
Initial time:	1.0 min.

Ramp: (°C/min.)	Final temp.: (°C)	Final Time (min.)
10.00	220	0
40.0	280	2.0

Run Time:	20.5 min.
Equilibration Time:	1.0 min.

MSD Operation Parameters:

Detector and transfer line:	280°C
Electron Multiplier (EM) range:	~2500 V
EM offset (V above Autotune):	200 V

SIM Settings:

Dichlobenil ions	m/z 171 (quantitation); m/z 173 (confirmation)
2,6-dichlobenil ions	m/z 173 (quantitation); m/z 175, 189 (confirmation)
Internal standard ions	m/z 205 (quantitation); m/z 207 (confirmation)
Resolution	Low
Dwell time	
dichlobenil and internal std	100 msec
2,6-dichlorobenzamide	50 msec
Solvent delay	13.0 min.

Retention times:

Dichlobenil	~10.9 min.
2,6-dichlorobenzamide	~15.2 min.
internal standard	~12.2 min.