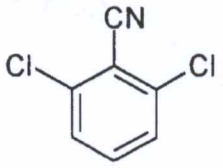
1.0 INTRODUCTION

PTRL Europe was contracted to validate an analytical method for the determination of dichlobenil and its metabolite 2,6-dichlorobenzamide (BAM) in soil with a target limit of quantification (LOQ of 0.010 mg/kg per analyte). The analytical method to be validated was provided by the Study Monitor and originates from Duphar B.V. (Laboratory Instructions RES 051 from 03-May-1991).

2.0 MATERIALS AND METHODS

2.1 TEST / REFERENCE ITEMS

Dichlobenil:



IUPAC name:	2,6-dichlorobenzonitrile
Molecular formula:	$C_7H_3Cl_2N$
Molecular weight:	172.0 g/mol (171/173 Cl_2 isotopic pattern)
CAS RN:	[*1194-65-6*]
Batch no.:	ARS-9108-BA
Expiration date:	31-Mar-2006
Purity:	100 %
Storage condition:	Frozen.

2,6-Dichlorobenzamide (BAM):

IUPAC name:	2,6-dichlorobenzamide
Molecular formula:	$C_7H_5Cl_2NO$
Molecular weight:	190.0 g/mol (189/191 Cl_2 isotopic pattern)
CAS RN:	[*2008-58-4*]
Batch no.:	ARS-81C25N
Expiration date:	31-Mar-2006
Purity:	100 %
Storage condition:	Frozen.

(Data as provided by the Sponsor, see Appendix 2 and 3 for further information).

2.2 TEST SYSTEM

Standard soil 2.2 (loamy sand) was obtained from LUFA Speyer (Germany) with full characterization (see Appendix 4).

2.3 CHEMICALS, EQUIPMENT, AND INSTRUMENTATION

2.3.1 Solvents and Chemicals

Acetone, ethyl acetate, methanol, petroleum ether (all HPLC or pesticide grade, Promochem)
Ethanol (99.5 %) (Merck)
Purified water (100 %) (Fresenius)
Ammonium chloride (NH_4Cl; 99.5 %) (Fluka)
Sodium chloride (NaCl; 99.5 %) (Fluka)
Sodium sulfate, anhydrous (Na_2SO_4; 99 %) (Merck)
Alumina SPE cartridge, neutral, 1000 mg (Baker)
Glass fiber filter GF8 (Schleicher&Schuell)
Folded filter (Schleicher & Schuell)

2.3.2 Laboratory Equipment

Analytical balance: RC210D (Sartorius)
Laboratory balance: L2200P (Sartorius)
Ultra Turrax T 50 (Janke & Kunkel)
SPE station Baker 12 G (Baker) and SPE 100 (Macherey & Nagel)
Buchner funnel (11 cm diameter)
Rotary evaporator Laborota 4002 (Heidolph), and RE-114 and RE-111 (Büchi)
Ultrasonic bath Transonic 460 and 700 (Elma)
Commonly used glassware and laboratory equipment

2.3.3 GC/ECD System

Varian CP-3380 GC/ECD gas chromatograph equipped with 8200 CX autosampler, CP 3380 GC with 1079 split/splitless-injector and electron capture detector, helium as carrier gas, and Varian Star GC-Workstation Software.

GC Column: Chrompack CP Sil 8 CB Low Bleed fused silica capillary column (30 m length, 0.32 mm inner diameter, 0.25 µm film thickness).

2.3.4 GC/MS System

Varian GC/MS system with 8100 Autosampler, 3400 GC, temperature programmed SPI injector, Varian Saturn 3 ion trap mass spectrometer with EI ionization.

GC Column: Chrompack CP Sil 8 CB Low Bleed fused silica capillary column (30 m length, 0.32 mm inner diameter, 0.25 µm film thickness).

2.4 STANDARDS

2.4.1 Stock Standard Solutions

Stock solutions were prepared in methanol as exemplified below:

	Purity [%]	Amount [mg]	Solvent Volume [mL]	Concentration [mg/mL]
Dichlobenil	100	20.00	20.0	1.00
2,6-Dichlorobenzamide (BAM)	100	20.00	20.0	1.00

2.4.2 Fortification Solutions

Fortification solutions were prepared in petroleum ether as exemplified below:

Solution [µg/mL]	Pipette [mL]	Dilute to [mL]	Obtain concentration [µg/mL]
Dichlobenil: 1000	0.20	20.0	Fortification solution used for 10xLOQ: 10
Dichlobenil: 10.0	2.0	20.0	Fortification solution used for LOQ: 1.0
2,6-Dichlorobenzamide (BAM): 1000	0.20	20.0	Fortification solution used for 10xLOQ: 10
2,6-Dichlorobenzamide (BAM): 10.0	2.0	20.0	Fortification solution used for LOQ: 1.0

2.4.3 Calibration Solutions

Calibration solutions of dichlobenil were prepared in petroleum ether as exemplified below:

Solution	Pipette [mL]	Dilute to [mL]	Obtain concentration [ng/mL]
Dichlobenil: 1.00 mg/mL	0.10	10.0	10000
Dichlobenil: 10000 ng/mL	0.10	1.0	1000
Dichlobenil: 10000 ng/mL	0.050	1.0	500
Dichlobenil: 10000 ng/mL	0.020	1.0	200
Dichlobenil: 10000 ng/mL	0.10	10.0	100
Dichlobenil: 10000 ng/mL	0.050	10.0	50
Dichlobenil: 100 ng/mL	2.0	10.0	20
Dichlobenil: 100 ng/mL	1.0	10.0	10
Dichlobenil: 100 ng/mL	0.50	10.0	5.0
Dichlobenil: 10 ng/mL	2.0	10.0	2.0
Dichlobenil: 10 ng/mL	1.0	20.0	1.0

Calibration solutions of 2,6-dichlorobenzamide (BAM) were prepared in ethyl acetate as exemplified below:

Solution	Pipette [mL]	Dilute to [mL]	Obtain concentration [ng/mL]
BAM: 1.00 mg/mL	0.10	10.0	10000
BAM: 10000 ng/mL	0.50	1.0	5000
BAM: 10000 ng/mL	1.0	10.0	1000
BAM: 10000 ng/mL	0.50	10.0	500
BAM: 10000 ng/mL	0.20	10.0	200
BAM: 10000 ng/mL	0.10	10.0	100
BAM: 1000 ng/mL	0.50	10.0	50
BAM: 1000 ng/mL	0.20	10.0	20
BAM: 1000 ng/mL	0.10	10.0	10
BAM: 1000 ng/mL	0.050	10.0	5.0

All solutions were prepared using volumetric flasks, volumetric pipettes and were stored in a refrigerator when not in use. The reference items were stable throughout the study period based on the comparison of chromatograms from analysis of standard solutions injected over the course of the study.

2.5 ANALYTICAL PROCEDURE

2.5.1 Method Outline

Soil (50 g) was extracted with a mixture of 0.2 % NH_4Cl aqueous solution / acetone / petroleum ether (17/75/75 v/v/v). The extract was filtered and after addition of water and sodium chloride, dichlobenil was partitioned into petroleum ether. Subsequently, 2,6-dichlorobenzamide (BAM) was partitioned into ethyl acetate. Aliquots of both extracts were purified on neutral alumina cartridges and the analytes were determined by capillary gas chromatography with electron capture detection (GC/ECD). Following concentration, the final extracts were analysed by GC/MS and GC/MS/MS (dichlobenil only) for demonstration of a confirmatory method.

2.5.2 Pretreatment of Standard Soil

The (almost dry) LUFA standard soil 2.2 was moistened by addition of 20 % of purified water to about 40 % of its maximum water capacity (50 g water for 100 g of dry weight of soil, according to documentation of standard soil, see Appendix 5).

2.5.3 Fortification Procedure

Fortification of untreated soil samples with dichlobenil and 2,6-dichlorobenzamide was performed to analyze method percent recoveries. Portions (50 g) of untreated soil material were fortified at 0.010 mg/kg of dichlobenil and 0.010 mg/kg 2,6-dichlorobenzamide (500 µL aliquots of 1.0 µg/mL fortification solutions) or at 0.10 mg/kg per analyte (500 µL aliquots of 10 µg/mL fortification solutions).

2.5.4 Extraction Procedure

The original analytical method approach from Duphar B.V. (Laboratory Instructions RES 051 from 03-May-1991) was used with some minor adapatations.

1. Homogenise the soil specimen and weigh 50 g of soil into a 400 mL beaker. Fortify as necessary.
2. Add 150 mL of acetone/petroleum ether (1:1 v/v) and 17 mL 0.2 % NH_4Cl-solution and blend for 5 min with Ultra Turrax T50 at 4000 rpm.
3. Filter through a Schleicher&Schuell glass fiber GF8 filter in a Buchner funnel into a 500 mL filtering flask.
4. Rinse the blender, beaker and the filter cake with approximately 30 mL of acetone/petroleum ether (1:1 v/v).
5. Transfer the extract into a 500 mL separatory funnel, add 300 mL of water and 10 mL of saturated NaCl-solution and shake for 1 minute. Allow the phases to separate and collect the petroleum ether-layer through a fluted filter, containing 20 g of anhydrous Na_2SO_4, into a 500 mL round bottom flask.

6. Add 100 mL of petroleum ether to the aqueous layer and shake for 1 min. Allow the layers to separate and collect the petroleum ether through the same fluted filter containing anhydrous Na_2SO_4.
7. Repeat extraction twice with 100 mL portions of petroleum ether and combine petroleum ether extracts. Keep the aqueous phase in a 600 mL beaker.
8. Evaporate at ambient temperature using a rotary evaporator until a volume of ≈ 30 mL remain and transfer the solution into a 50 mL volumetric flask and make-up to volume with petroleum ether. This is the Dichlobenil extract A.
9. Add 100 mL of ethyl acetate to the aqueous phase of step 7 and shake for 1 min. Allow the phases to separate and collect the ethyl acetate through a fluted filter containing 20 g of anhydrous Na_2SO_4, into a 500 mL round bottom flask.
10. Repeat extraction twice with 100 mL portions of ethyl acetate and combine ethyl acetate extracts.
11. Evaporate to dryness using a rotary evaporator and dissolve the residue in 10.0 mL of acetone/petroleum ether (1:9 v/v). This is the 2,6-dichlorobenzamide (BAM) extract B.

2.5.5 Clean-up Procedure - Dichlobenil

1. Condition an alumina cartridge with 5 mL of petroleum ether.
2. Bring 5.0 mL of dichlobenil extract A onto the cartridge.
3. Wash with 1 mL of petroleum ether.
4. Elute with 8 mL of 2 % acetone/petroleum ether into a 10 mL volumetric flask and make-up to volume with 2 % acetone/petroleum ether. This is the final extract A for GC/ECD analysis of dichlobenil.
5. 5.0 mL of the GC/ECD final extract A were concentrated in a gentle stream of nitrogen to ≈ 0.3 mL. By addition of petroleum ether the final volume for GC/MS analysis of dichlobenil was adjusted to 0.5 mL (concentration factor: 10, theoretical final volume: 1.0 mL).

2.5.6 Clean-up Procedure – 2,6-Dichlorobenzamide (BAM)

1. Condition an alumina cartridge with 5 mL of petroleum ether.
2. Bring 5.0 mL of 2,6-dichlorobenzamide extract B onto the cartridge.
3. Wash twice with 5 mL portions of acetone/petroleum ether (1:9 v/v).
4. Elute with 7 mL of ethanol/petroleum ether (15:85 v/v).
5. Evaporate the solvents at 40°C and dissolve the residue in 5.0 mL of ethyl acetate. This is the final extract B for GC/ECD analysis of 2,6-dichlorobenzamide.
6. 3.0 mL of the GC/ECD final extract B were concentrated in a gentle stream of nitrogen to ≈ 0.4 mL. By addition of ethyl acetate the concentrated extract was adjusted to a final volume of 0.6 mL for GC/MS analysis of 2,6-dichlorobenzamide (concentration factor: 5, theoretical final volume: 1.0 mL).

2.6 INSTRUMENTAL ANALYSIS

The final extracts were analyzed by gas chromatography on a capillary column with a non-polar Chrompack Sil-8 stationary phase using electron capture detection (GC/ECD). The quantitative determination was carried out by external standardization.

2.6.1 GC/ECD Conditions

The following GC/ECD method was used for identification and quantification of dichlobenil and 2,6-dichlorobenzamide respectively in the final extracts A and B:

GC/ECD System:	Varian CP-3380 GC/ECD gas chromatograph equipped with 8200 CX autosampler, CP-3380 GC with 1079 split/splitless-injector and electron capture detector, and Varian Star GC-Workstation Software.
Autosampler:	Solvent flush sampling, solvent plug size 1.0 µL with lower and upper air gap (solvent: petroleum ether for dichlobenil analysis, ethyl acetate for 2,6-dichlorobenzamide analysis), injection volume 1 µL, injection rate 1 µL/s.
Injection technique	Split/splitless injection: 2.0 min splitless time. Injector temperature: 230 °C.
Carrier gas:	Helium at 10 psi constant pressure.
GC column:	Varian Chrompack CP Sil 8 CB Low Bleed (equivalent to J&W Scientific DB-5): 30 m length, 0.32 mm i.d., 0.25 µm film thickness.
Oven program:	Initial temperature 70 °C, hold 2.0 min, rate 20 °C/min to 270 °C, hold for 2 min at 270 °C. Retention time (Dichlobenil): 7.4 min (≈ 180°C). Retention time (2,6-dichlorobenzamide): 9.9 min (≈ 230°C).

2.6.2 GC/ECD Analysis of Specimen Extracts

For analysis of dichlobenil calibration functions ranging from 1.0 ng/mL to 100 ng/mL (7 levels) with 1-µL injections were established by injecting calibration solutions interspersed with final extracts. Calibration functions for the GC/ECD analysis of 2,6-dichlorobenzamide (BAM) ranged from 5 ng/mL to 1000 ng/mL (8 levels). Peak areas were used for evaluation.

For both analytes the calibration functions were calculated and plotted by linear regression. The correlation coefficients R^2 were always > 0.99. Representative calibration functions used to evaluate final extracts are given in Figure 1 (dichlobenil) and Figure 8 (2,6-dichlorobenzamide) respectively. GC/ECD chromatograms of calibration solutions are shown in Figure 2 to Figure 4 (dichlobenil) and Figure 9 to Figure 11 (2,6-dichlorobenzamide) respectively. GC/ECD chromatograms in Figure 5 to Figure 7 (dichlobenil) and Figure 12 to Figure 14 (2,6-dichlorobenzamide) demonstrate results obtained from final extracts of fortified (10xLOQ and LOQ) and blank control specimens.

Repeatability of GC/ECD determination for both dichlobenil and 2,6-dichlorobenzamide was demonstrated by duplicate injections of selected specimen extracts of each fortification level.

2.7 CALCULATIONS

2.7.1 Calculation of Residue

Concentrations of dichlobenil and 2,6-dichlorobenzamide (BAM) in soil specimen extracts (c_{End}, expressed in ng/mL) were calculated by the Varian Star chromatographic software using the calibration function.

Dichlobenil:

The residues R (in mg/kg) of dichlobenil in soil specimens were calculated by Microsoft ® Excel 2000 using the (average) c_{End} obtained and the following equation:

$R_{found} = c_{End} \times (V_{R1} \times V_{End}) / (V_{R2} \times W_{Sample} \times 1000)$
$= c_{End} \times$ Multiplier M

where:

R_{found} = Residue of dichlobenil in soil specimen, mg/kg

c_{End} = (Average) concentration of dichlobenil in the final extract, in ng/mL

V_{R1} = Volume of dichlobenil raw extract in petroleum ether, 50 mL

V_{R2} = Volume of extract used for alumina SPE clean-up, 5.0 mL

V_{End} = Final volume of extract, 10.0 mL

W_{Sample} = Weight of soil specimen used for extraction, 50 g

1000 = Factor for conversion of µg/kg into mg/kg

Multiplier M = Multiplier used to calculate R_{found} from c_{End}

See Table 1 for GC/ECD result evaluation and calculations for dichlobenil.

2,6-Dichlorobenzamide (BAM):

The residues R (in mg/kg) of 2,6-dichlorobenzamide (BAM) in soil specimens were calculated using the following equation:

$R_{found} = c_{End} \times (V_{R1} \times V_{End}) / (V_{R2} \times W_{Sample} \times 1000)$
$= c_{End} \times$ Multiplier M

where:

R_{found} = Residue of 2,6-dichlorobenzamide (BAM) in soil specimen, mg/kg

c_{End} = (Average) concentration of 2,6-dichlorobenzamide (BAM) in the final extract, in ng/mL

V_{R1} = Volume of 2,6-dichlorobenzamide (BAM) raw extract in acetone / petroleum ether (1:9 v/v), 10.0 mL

V_{R2} = Volume of extract used for alumina SPE clean-up, 5.0 mL

V_{End} = Final volume of extract, 5.0 mL

W_{Sample} = Weight of soil sample used for extraction, 50 g

1000 = Factor for conversion of µg/kg into mg/kg

Multiplier M = Multiplier used to calculate R_{found} from c_{End}

See Table 1 for GC/ECD result evaluation and calculations for 2,6-dichlorobenzamide (BAM).

2.7.2 Recoveries

Recoveries (Rec.) were calculated as follows:

Rec = $(R_{found} / R_{fortified}) * 100\ \%$

2.7.3 Example

An example calculation for the recovery of dichlobenil and 2,6-dichlorobenzamide (BAM) in a soil specimen fortified at 0.10 mg/kg (10xLOQ) is shown below (see Table 1 for summary, Appendix 5 (dichlobenil) and Appendix 6 (2,6-dichlorobenzamide) for evaluation tables, and Figure 5 (dichlobenil) and Figure 12 (2,6-dichlorobenzamide) for chromatograms):

Dichlobenil:

The soil specimen with the PTRL sample ID P556-44A was injected twice from a final volume V_{End} of 10 mL. External calibration using the calibration function as depicted in Figure 1 gave an average final concentration c_{End} of 48.12 ng/mL.

Thus:

R_{found} = $c_{End} \times (V_{R1} \times V_{End}) / (V_{R2} \times W_{Sample} \times 1000)$
= c_{End} x Multiplier M
= 48.12 ng/mL x (50 mL x 10 mL) / (5.0 mL x 50 g x 1000)
= 48.12 ng/mL x 0.0020 mL/g
= 0.096 mg/kg

and:

Rec = $(R_{found} / R_{fortified}) * 100\ \%$
= (0.096 mg/kg / 0.10 mg/kg) * 100 % = 96 %.

2,6-Dichlorobenzamide (BAM):

The soil specimen with the PTRL sample ID P556-44B was injected twice from a final volume V_{End} of 5.0 mL. External calibration using the calibration function as depicted in Figure 8 gave an average final concentration c_{End} of 364.35 ng/mL.

Thus:

R_{found} = $c_{End} \times (V_{R1} \times V_{End}) / (V_{R2} \times W_{Sample} \times 1000)$
= c_{End} x Multiplier M
= 364.36 ng/mL x (10 mL x 5.0 mL) / (5.0 mL x 50 g x 1000)
= 364.36 ng/mL x 0.00020 mL/g
= 0.073 mg/kg

and:

Rec = $(R_{found} / R_{fortified}) * 100\ \%$
= (0.073 mg/kg / 0.10 mg/kg) * 100 % = 73 %.

2.8 GC/MS Confirmatory Method

The intense molecular ion chlorine isotopic pattern with signals at 171 (also used for quantification) and 173 m/z was used for GC/MS confirmation of dichlobenil residues (see Figure 15). Residues of dichlobenil in the full scan electron impact (EI) mass spectrum were further confirmed by highly specific GC/MS/MS detection of the daughter ion signal at 136 m/z.

Residues of the metabolite 2,6-dichlorobenzamide (BAM) were confirmed by GC/MS detection of the intense fragment ions at 173 (also used for quantification), 175 and 189 m/z.

2.8.1 GC/MS and GC/MS/MS Conditions

GC/MS System:	Varian Saturn III GC/MS with equipped with 8100 autosampler, 3400 GC with SPI injector and ion trap mass spectrometer, helium as carrier gas, and Varian GC/MS Software.
Autosampler:	Solvent flush sampling, solvent plug size 0.5 µL with lower and upper air gap, injection volume 3 µL, injection rate 1 µL/s.
Injection technique	Septum equipped temperature programmable (SPI) injector. Injector temperature: 230 °C.
Carrier gas:	Helium at 8 psi constant pressure.
GC column:	Varian Chrompack CP Sil 8 CB Low Bleed (equivalent to J&W Scientific DB-5): 30 m length, 0.32 mm i.d., 0.25 µm film thickness.
Oven program:	Initial temperature 70 °C, hold 2.0 min, rate 20 °C/min to 270 °C, hold for 2 min at 270 °C. Retention time (dichlobenil): ≈ 5.5 min (140 °C). Retention time (2,6-dichlorobenzamide): ≈ 7.7 min (184 °C).
MS detection:	*Dichlobenil:* Narrow mass range GC/MS detection, scan range 168 to 178 m/z, mass defect -21 mu/100 u, filament current 40 µA. Ions at 171 m/z (quantification) and at 173 m/z (confirmation) were used for specific detection of dichlobenil residues. *2,6-Dichlorobenzamide:* Narrow mass range GC/MS detection, scan range 168 to 195 m/z, mass defect -21 mu/100 u, filament current 40 µA. Ion at 173 m/z (quantification) , ions at 175 m/z and 189 m/z were used for confirmation of 2,6-dichlorobenzamide residues.
MS/MS detection	*Dichlobenil:* Parent ion 171 m/z, mass isolation window 3 m/z. Resonant collision induced dissociation (CID), 1.0 V amplitude, 60 m/z storage level. Daughter ion scan range 80 to 180 m/z. The daughter ion present at 136 m/z $(M\text{-}Cl)^+$ was used for confirmation of dichlobenil residues.

2.8.2 GC/MS and GC/MS/MS Confirmatory Analysis of Specimen Extracts

Selected specimen extracts (one blank control, one LOQ and one 10xLOQ fortification) were analysed to establish confirmatory methods for both analytes (see Appendix 7 to Appendix 9 for evaluation tables).

For analysis of dichlobenil GC/MS and GC/MS/MS linear calibration functions ranging from 10 ng/mL to 1000 ng/mL (6 levels) with 3-µL injections were established by injecting calibration solutions interspersed with final extracts. Linear calibration functions for the GC/MS analysis of 2,6-dichlorobenzamide (BAM) ranged from 20 ng/mL to 5000 ng/mL (6 levels). Correlation coefficients R for all calibration curves were always > 0.99.

For both analytes the calibration functions were calculated and plotted by linear regression.

Representative calibration functions used to evaluate final extracts are given in Figure 16 (GC/MS of dichlobenil), Figure 23 (GC/MS/MS of dichlobenil), and Figure 29 (GC/MS of 2,6-dichlorobenzamide).
Chromatograms of calibration solutions are shown in Figure 17 to Figure 19 (GC/MS of dichlobenil), Figure 24 and Figure 25 (GC/MS/MS of dichlobenil), and Figure 30 to Figure 32 (GC/MS of 2,6-dichlorobenzamide).
Chromatograms of selected soil specimen extracts are presented in Figure 20 to Figure 22 (GC/MS of dichlobenil), Figure 26 to Figure 28 (GC/MS/MS of dichlobenil), and Figure 33 to Figure 35 (GC/MS of 2,6-dichlorobenzamide).

2.9 TIME REQUIRED FOR ANALYSIS

Time required per set of approx. 6 specimens (12 final extracts) for extraction, partition, alumina clean-up is 14 person-hours. A GC/ECD run requires approximately 20 min, thus a set of 6 specimens (12 final extracts) and the required number of calibration injections needs approximately 10 instrument-hours.

Thus, the time period from the initiation of extraction until completion of instrumental analysis, including evaluation amounts to approximately 28 person-hours.