ABSTRACT

An analytical method for the determination of cyanazine and its metabolites in soil is described. The method is based on extraction of the soil using a methanol/water solvent and sonic disruption. Metabolites that have become hydrogen-bonded to the soil matrix are extracted by a second treatment using methanol/0.1 N NaOH and sonic disruption. Quantitation of the extracts is done by HPLC, based on the use of RP8/Anion "Mixed Mode" and ODS columns with ultraviolet detection. Procedural recoveries range from 75 to 105%; the quantitation limit is 0.05 ppm for each metabolite, based on a 5 g sample.

INTRODUCTION

An analytical method based on the use of liquid chromatography is described for cyanazine and its four metabolites in soil. These metabolites result from hydrolysis of the parent compound, which is the active ingredient in Bladex® Herbicide. The first metabolite formed is the amide of cyanazine, IN-BV453

(formerly coded as SD 20258). The amide functionality is further hydrolyzed to the chloro acid metabolite, IN-BV424 (formerly coded as SD 20196). The chloro functionality is then hydrolyzed to yield the ethylated hydroxy acid metabolite, IN-CH881 (formerly coded as SD 31223). Finally, the ethyl group is lost, resulting in the de-ethylated hydroxy acid metabolite, IN-CH882 (SD 31224). See Figure 1 for structures.

The method is based on extraction of the parent compound, IN-BV453, and IN-BV424 using 50/50 methanol/water. Some of the hydroxy acids, IN-CH881 and IN-CH882 are also extracted. The sample is subsequently extracted with 50/50 methanol/0.1 N NaOH for determination of the remainder of the unbound hydroxy acids. Quantitation of the extracts is done by HPLC, based on the use of RP8/Anion "Mixed-Mode" and ODS columns with UV detection. The quantitation limit is 0.05 ppm for each compound, based on a 5 g sample.

REAGENTS AND APPARATUS

Analytical Standards - Cyanazine (DPX-R1957), IN-BV453, IN-BV424, IN-CH881, and IN-CH882 were provided by E. I. DuPont de Nemours & Co., Inc., Agricultural Products Department, Experimental Station, Wilmington, Delaware.

Solvents - Methanol (MX0488-1), acetonitrile (AX0142-1), and ethyl acetate (EX0241-1) all HPLC grade, were all provided by E. M. Science, Cherry Hill, N. J.

Solutions - 0.1 N NaOH for the second set of extractions is prepared by dissolving 8 g of 50% NaOH solution (J. T. Baker

Chemical Co., Phillipsburg, NJ, catalog no. 3727-01) in 1 L of distilled deionized water obtained from a Milli-Q® water purification system (Millipore Corporation, Bedford, MA).

The phosphate buffers used in the SCX cleanup step are pH 1.8, 100 mM sodium phosphate and pH 1.8, 25 mM sodium phosphate. The 100 mM buffer is prepared by dissolving 13.8 g of sodium phosphate monohydrate (EM Science, Catalog No. SX0710-1) in 1 L of distilled deionized water. The pH is then adjusted to 1.8 by addition of HPLC grade phosphoric acid (J. T. Baker Co., O260-01). The 25 mM buffer is prepared by dissolving 3.45 g of sodium phosphate in 1 L of distilled deionized water, and adjusting the pH to 1.8 by addition of HPLC grade phosphoric acid.

The HPLC mobile phase buffers are pH 3.75, 2 mM sodium acetate and pH 3.75, 5 mM sodium acetate. The pH 3.75, 2 mM buffer is prepared by dissolving 270 mg of sodium acetate (EM Science, catalog no. SX0255-1) in 1 L of distilled deionized water, and adjusting the pH to 3.75 by addition of HPLC grade glacial acetic acid (EM Science, AX0073-1). The 5 mM buffer is prepared by dissolving 680 mg of sodium acetate in 1 L of distilled deionized water, and adjusting the pH to 3.75 by addition of HPLC grade glacial acetic acid.

Sonic Disruptor - A Braunsonic® Ultrasonifier (B. Braun, Inc, Burlingame, CA) was used to extract the soil samples. An intermediate size probe (B. Braun, catalog no. 853813) equipped with a cooling jacket (B. Braun, catalog no. 853816) was used for this work.

Centrifuge - RC5C Sorval® centrifuge (DuPont Co., Biomedical Products, Glasgow, DE) with a 7000 rpm maximum rotor (P/N HS-4) or a 20,000 rpm maximum rotor (P/N SS-34).

Centrifuge Bottles - 250 mL polypropylene wide mouth bottles (VWR Scientific, Bridgeport, NJ, catalog no. 16129-028).

Centrifuge Tubes - 40 mL polypropylene 29 x 103 mm tubes (VWR Scientific, catalog no. 21007-303).

Ultrasonic Bath - Bransonic ultrasonic cleaner (Bransonic Co., Danbury, CT, catalog no. B2200R-4).

Rotary Vacuum Evaporator - For concentration of samples, a Rotovapor-R (Brinkman Instruments, Westbury, NY) was operated at 26 inches of mercury with the flask in a 50 C water bath. Pear-shaped flasks (Kontes Glass, Vineland, NJ, catalog no. K-608700) were used.

Nitrogen Evaporator - An N-EVAP (Organomation Associates, Worcester, MA) was used to concentrate the samples to their final volume under a stream of nitrogen.

pH meter - The meter used to determine pH was a Corning Model 650, obtained from Fisher Scientific Co., Pittsburgh, PA.

Ion Exchange Cleanup - The strong cation ion exchange resin was AGMP50 resin (Bio-Rad Laboratories, Richmond, CA, catalog no. 143-0851). The resin was packed into empty 8 mL cartridges obtained from Analytichem International, Harbor City, CA (catalog no. 600820). The vacuum elution system was obtained from Supelco Inc., Bellefonte, PA (catalog no. 5-7030).

Liquid Chromatography System - A Hewlett-Packard 1090 LC equipped with an autosampler, diode-array detector, and a series "M" data handling computer system was used. The operational parameters

are given in Appendices 1 and 2. The column was either a "Mixed Mode" RP8/Anion column, 25 cm x 4.6 mm i.d. (Alltech Associates, Deerfield, IL, catalog no. 12369) or a Partisil 5 ODS 3 column, 25 cm x 4.6 mm i.d. (Alltech Associates, catalog no. 4238001). In order to prolong the lifetime of the column, a presaturation column (Alltech Associates, catalog no. 28610), and a guard column cartridge holder (Alltech, catalog no. 28013) equipped with an RP8/anion cartridge (Alltech, catalog no. C8800) or a C8 cartridge (Alltech, catalog no. 28017) were used.

EXPERIMENTAL

Processing Of Samples

Preprocessing - The procedures detailed in Du Pont Study No. AMR-525-86 (See Appendix 3) are to be followed for the moisture content determination, drying, and homogenization of the soil sample. Consult study protocol for instructions on segmenting cores and compositing of samples.

Methanol/Water Extractions - (Note: steps 2-10 may be skipped if, based upon prior analyses, it is certain that the parent, amide, and chloro acid metabolites are no longer present in the sample, and only the Hydroxy acid metabolites remain).

1. Weigh 5 g of the homogenized soil sample into a 250 mL polypropylene centrifuge bottle.
2. Add 50 mL of 50/50 (v/v) methanol/distilled deionized water to the centrifuge bottle. Extract using the sonic disruptor set at 300 W for 4 min.
3. Centrifuge the sample at 7000 rpm for 10 min.

4. Decant the supernatant into a rotary evaporator flask.

5. Repeat steps 2-4 two more times, combining the supernatant solutions in the rotary evaporator flask. Save the remaining solids for the second set of extractions.

6. Evaporate the combined supernatants to a volume of approximately 20 ml.

7. Quantitatively transfer the evaporated supernatant solution to a 40 mL centrifuge tube. Use ~5 mL of water to rinse the flask. Centrifuge at 20,000 rpm for 10 min. Decant back into the rotovap flask and continue evaporating to a volume of approximately 2-3 ml.

8. Transfer the solution into a 10 mL graduated centrifuge tube, and use 2-3 mL of water to rinse the rotovap flask. Add the rinsates to the centrifuge tube.

9. Evaporate the sample solution to a volume of less than 1 mL using the N-EVAP with a steady stream of nitrogen and the water bath set at 60°C.

10. Use a disposable pipet to transfer the solution to a 1 mL volumetric flask and bring to a final volume of 1 mL with water. This sample is now ready for HPLC analysis, and is stable for three days if kept refrigerated.

<u>Methanol/0.1 N NaOH Extraction</u>

11. Add 50 mL of 50/50 (v/v) methanol:0.1 N NaOH to the solids remaining from step 5. Extract using the sonic disruptor set at 300 W for 4 min.

12. Centrifuge the sample at 7000 rpm for 10 min.

13. Decant the supernatant into a rotary evaporator flask.

14. Repeat steps 11-13 two more times, combining the supernatant solutions in the rotary evaporator flask.

15. Evaporate the methanol from the sample on the rotary evaporator, so that 75 mL or less remains.

16. Transfer the remaining solution to a 250 mL beaker. Rinse the flask with ca. 10 mL of water and add the rinsate to the beaker. Adjust the pH to 2.0 by addition of concentrated HCl.

17. Transfer the solution to a 250 mL separatory funnel.

18. Add 50 mL ethyl acetate, shake for 1 minute, aspirate off the ethyl acetate (top) layer, and discard. (The upper layer can be aspirated through use of a disposable pipet connected to a vacuum flask by a length of rubber tubing.)

19. Repeat step 18 two more times. Save the aqueous layer. This sample may be kept for 24 hours if refrigerated.

<u>Ion-Exchange Cleanup</u>

20. The empty cartridges specified in the Reagents and Apparatus section above have two frits at the bottom of the cartridge. Use a disposable pipet to push the frits out of the cartridge through the wider opening. Replace one of the frits back into the bottom of the cartridge.

21. Weigh out 1 g of the cation exchange resin into a small beaker. Slurry the resin in about 10 mL of pH 1.8, 100 mM phosphate buffer, and pour into the cartridge while applying gentle vacuum through use of the vacuum manifold. Rinse any resin remaining in the beaker into the cartridge using ca.5 mL volumes of buffer. Do not allow the resin

bed to dry out until all of the resin has been transferred into the column. Press the other frit gently onto the resin bed.

22. Pre-elute the cartridge (under vacuum) with two 5 mL volumes of 50/50 (v/v) methanol:concentrated ammonium hydroxide. Allow all of the liquid to elute from the cartridge between rinses.

23. Pre-elute the cartridge with two 5 mL volumes of pH 1.8, 100 mM phosphate buffer.

24. Repeat steps 22 and 23 one more time.

25. Attach a 100 mL reservoir to the top of the cartridge. Pour the aqueous sample extract obtained in step 19 into the reservoir, and pass the sample through the column into a waste container inside the vacuum manifold at a flow rate of 3-5 ml/min.

26. Rinse the cartridge with three 5 mL portions of the pH 1.8, 100 mM phosphate buffer, followed by two 5 mL portions of water. These rinses also may drain into the waste container.

27. Place a 10 mL graduated centrifuge tube containing two drops of glycerin into the vacuum manifold, under the cartridge outlet.

28. Elute the cartridge with two 2 mL portions of 50/50 (v/v) methanol:concentrated ammonium hydroxide at a flow rate of 3-5 ml/min.

29. Evaporate the eluate collected in the centrifuge tube to "dryness" (i.e., only the glycerin remains) using the

N-EVAP with the water bath set at 70°C. Bring up to the 1 mL mark with pH 1.8, 25 mM phosphate buffer, mixing thoroughly on a Vortex mixer to dissolve the glycerin and rinse down the walls of the tube. The sample is now ready for HPLC analysis, and is stable for three days.

PREPARATION OF STANDARDS

The stock standard solutions are prepared by dissolving, accurately weighed, 10.0 mg of cyanazine and its metabolites in 100 mL of 50/50 (v/v) methanol:water. It is recommended that separate stock solutions be prepared for each compound, since they could then be used to identify retention times uniquely if a question ever arises. These solutions are quite stable, and can be stored up to six months in a refrigerator.

A 10 ug/mL mixed solution of the standards is prepared by pipetting 10 mL of each stock solution into a clean, dry 100 mL volumetric flask and diluting to volume with water. This standard is stable for one week, if kept refrigerated. Standards for HPLC analysis with concentrations of 0.25, 0.5, 0.75, and 1.0 ug/mL are prepared by pipetting 0.25, 0.5, 0.75, and 1 mL of the 10 ug/mL mixed standard into each of four 10 mL volumetric flasks, and diluting to volume with water. These standards should be prepared daily.

FORTIFICATIONS

A control (untreated) sample fortified with cyanazine and its four soil metabolites is run with each set of samples that are

extracted with methanol/water. Samples fortified at the 0.05, 0.1, or 0.2 ppm levels are prepared by pipetting 0.25, 0.5, or 1 mL of a 1 ug/mL mixed standard (prepared as described above) of the five compounds over the surface of the control sample after it is weighed into the centrifuge bottle, before any extracting solution is added.

A control (untreated) sample fortified with the two hydroxy acid metabolites (IN-CH881 and IN-CH882) are run with all samples when they are extracted with methanol/0.1 N NaOH. Samples fortified at the 0.05, 0.1, and 0.2 ppm levels are prepared by pipetting 0.25, 0.5, and 1 mL of a 1 ug/mL mixed standard (prepared as described above, using only the hydroxy acid stock solutions) of the two hydroxy acids over the surface of the control sample after it is weighed into the centrifuge bottle, before any extracting solution is added.

HPLC ANALYSES

There are two sets of HPLC conditions in this method. The hydroxy acid metabolites (IN-CH881 and IN-CH882) must be determined using the mixed-mode column, while the chloro acid metabolite (IN-BV424) must be determined using the ODS column. Cyanazine and its amide metabolite (IN-BV453) may be determined using either column. The methanol/water extractions should be analyzed using both methods, while the methanol/0.1 N NaOH extractions need only be analyzed using the mixed mode column.

Analysis Using ODS Column

Operational parameters for the determination of cyanazine and its amide and chloro acid metabolites are given in Appendix 1.

A more conventional listing of the gradient conditions is as follows:

Time (minutes)	% Acetonitrile	% pH 3.75, 5 mM Sodium Acetate
0	20	80
10	20	80
20	50	50

Sample chromatograms of a control sample and a control sample fortified at the 0.05 ppm level with the three compounds are shown in Figure 2. After use, the column is first flushed with 80/20 (v/v) water:acetonitrile, then with acetonitrile. The column should be flushed with acetonitrile before storage.

Analysis Using RP8/Anion Mixed Mode Column

Operational parameters for the determination of cyanazine, the amide metabolite, and the two hydroxy acid metabolites are given in Appendix 2. A more conventional listing of the gradient conditions is as follows:

Time (minutes)	% Acetonitrile	% pH 3.75, 5 mM Sodium Acetate
0	10	90
10	10	90
20	30	70
25	90	10
30	90	10

Sample chromatograms of a control sample and a control sample fortified at the 0.05 ppm level with the four compounds are shown in Figure 3. Similarly, chromatograms of a control sample and a sample fortified with 0.05 ppm of the two hydroxy acids, then taken through the methanol/0.1 N NaOH extraction and ion-exchange cleanup procedures, are shown in Figure 4. If all four compounds are to be

determined by this method, it is recommended to monitor the UV absorbance at 235 nm. However, if only the hydroxy acids are to be determined, monitoring at 245 nm will give less baseline rise and better sensitivity. After use, the column is first flushed with water, then with 50/50 (v/v) methanol/water.

Calibration and Calculations

Inject the four standards and each of the samples. Prepare a calibration curve by plotting the peak height of each compound vs its concentration in ug/ml. If all four standards show a reasonable fit to a straight line through the origin, calculate the response factor (RF) for each standard:

$$RF = \text{peak ht (mm)} \; / \; \text{ug injected}$$

where ug injected is given by:

$$\text{ug injected} = \text{concentration of standard (ug/ml)} \times \text{volume injected (ml)}$$

The amount of cyanazine or any of its metabolites in a given sample can be calculated from the following equation:

$$\text{ug/g (ppm)} = \frac{H(E)}{RF(S)W}$$

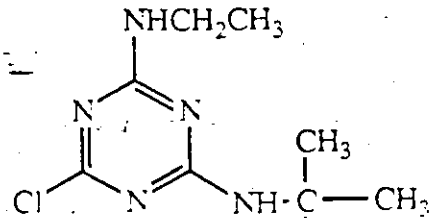
where H is the peak height in mm, RF is the response factor in mm/ug, E is the final volume of the sample extract in ml, S is the volume of the sample extract injected in ml, and W is the sample weight in g.

If the hydroxy acid metabolites, CH881 and CH882, are found in both extracts of any particular sample, the levels of these two

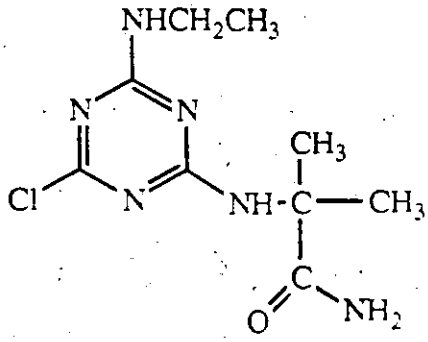
compounds are reported as the sum of the two results. Therefore, if these metabolites are detected at levels above 0.01 ppm in any extract, this result is summed with the levels found in the other extract. If the sum of the levels found in the two extracts is >0.05 ppm, report this sum. If it is < 0.05 ppm report it as such.

FIGURE 1

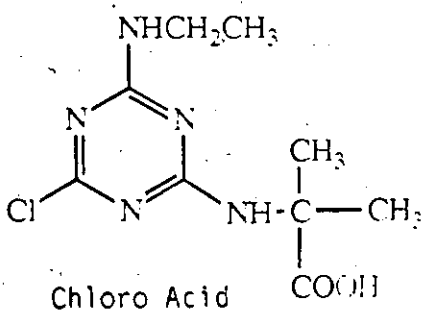
Structures Of Cyanazine and its Soil Metabolites



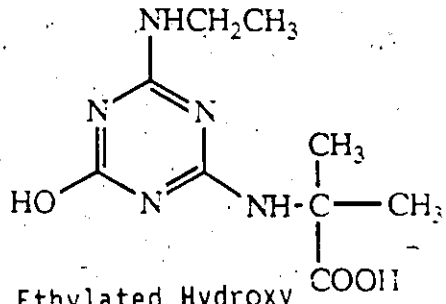
Cyanazine
DPX-R1957
(SD 15418)



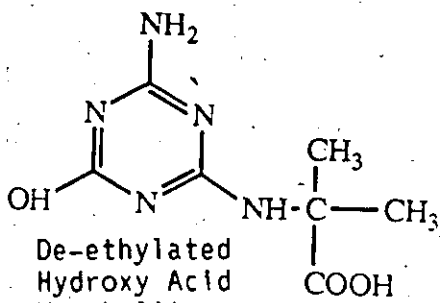
Amide Metabolite
IN-BV453
(SD 20258)



Chloro Acid
Metabolite
IN-BV424
(SD 20196)



Ethylated Hydroxy
Acid Metabolite
IN-CH881
(SD 31223)



De-ethylated
Hydroxy Acid
Metabolite
IN-CH882
(SD 31224)

APPENDIX 1

HPLC Operational Parameters for Analyses Conducted Using RP8/Anion "Mixed Mode" Column

L I Q U I D C H R O M A T O G R A P H initial parameters

Flow :	2.000 ml/min	
Solvent A :	10.0 % ACN	
B :	90.0 % pH 3.75 2 mM NaOAc	
C :	0.0 % WATER	
Oven Temperature :	45.0 C	
Max Pressure :	400 bar	Min Pressure : off
Stop Time :	30.10 min	
Post Time :	7.00 min	Contacts : 0000
Injection Volume :	100.0 ul	Slowdown : 2

L I Q U I D C H R O M A T O G R A P H timetable

Time (min)								
10.00	Solvent	A :	10.0 %	B :	90.0 %	C :	0.0 %
20.00	Solvent	A :	30.0 %	B :	70.0 %	C :	0.0 %
25.00	Solvent	A :	90.0 %	B :	10.0 %	C :	0.0 %

I N J E C T O R P R O G R A M

Slowdown Draw & Eject :	5
Min :	2
Hold after Draw & Eject :	0

0.0 ul accumulated in Syringe with Line# 0

Line#	Function
1	Inject

D I O D E - A R R A Y D E T E C T O R signals & spectra

SIGNALS	A	B	C	D	E	F	G	H
Sample (nm)								
Wavelength :	235	245	off	off	off	off	off	off
Bandwidth :	10	10						
Reference (nm)								
Wavelength :	550	550						
Bandwidth :	100	100						

APPENDIX 2

HPLC Operational Parameters for
Analyses Conducted Using ODS Column

L I Q U I D C H R O M A T O G R A P H — initial parameters

Flow :	2.000	ml/min
Solvent A :	20.0	% ACN
B :	80.0	% pH 3.75 5 mM NaOAc
C :	0.0	% WATER
Oven Temperature :	45.0	C
Max Pressure :	400	bar
Min Pressure :	off	
Stop Time :	20.10	min
Post Time :	7.00	min
Contacts :	0000	
Injection Volume :	100.0	ul
Slowdown :	1	

L I Q U I D C H R O M A T O G R A P H — timetable

Time (min)								
10.00	Solvent	A :	20.0 %	B :	80.0 %	C :	0.0 %
20.00	Solvent	A :	50.0 %	B :	50.0 %	C :	0.0 %

I N J E C T O R P R O G R A M

Slowdown Draw & Eject :	5
Mix :	2
Hold after Draw & Eject :	0

0.0 ul accumulated in Syringe with Line# 0

Line#	Function
1	Inject

D I O D E - A R R A Y D E T E C T O R — signals & spectra

SIGNALS	A	B	C	D	E	F	G	H
Sample (nm)								
Wavelength :	235	off	off	off	off	off	off	off
Bandwidth :	10							
Reference (nm)								
Wavelength :	550							
Bandwidth :	100							

APPENDIX 3

Soil Preprocessing Procedures
Excerpted From Du Pont Study No. AMR-525-86

Appendices I, II, or III for suggested methods of comminuting crop RAC's, animal RAC's, or processed commodities, respectively. Note: Some pesticides, e.g., dithiocarbamates, are extremely vulnerable to enzymatic decomposition once a sample is comminuted. Observe any precautions in the protocol.

3.3.5 Soils.

3.3.5.1 Prepare all soils for analysis according to the following procedure:

a. Remove rocks, pebbles, and plant debris.
b. Assess the moisture content of a subsample and record the percent moisture (see 3.3.5.2).
c. Dry the entire sample to visual dryness on trays lined with foil (for example stainless steel trays lined with aluminum foil) at room temperature for 24-48 hours, stirring the sample periodically. (Do not dry samples beyond the air-dry state as decomposition or irreversible adsorption of residue may result.)
d. Ball mill the sample using either (1) masonry jars* with ceramic ball bearings on a roller (or equivalent apparatus) or (2) Turbula in plastic jugs until the sample is visually homogeneous. (*Decontaminate masonry jars: Soak in dilute bleach solution (Clorox) overnight. Scrub thoroughly. Rinse with 1:1 methanol:isopropanol, then methylene chloride, then 1:1 methanol:isopropanol)
e. Remove a subsample for analysis and freeze the remainder.

3.3.5.2 Determining the moisture content in a soil sample.

a. Calibrate and zero-balance to true zero. This should be done on each day weights are taken.
b. Label and weigh aluminum pan. DO NOT TOUCH TARE KNOB AT ANY TIME DURING WEIGHING PROCEDURE. This would invalidate results.
c. Record weight of aluminum pan (empty) as tare weight.
d. Place an appropriate amount of soil (usually 3 to 6 g) in weigh boat.
e. Record weigh boat and soil as tare and wet material.

APPENDIX 3
(continued)

f. Dry for at least 5 hours in a 103°C ± 5° oven. Note: Overnight drying is preferred, and drying for extended periods is acceptable as long as the sample itself does not degrade or lose weight under 103°C heat.
g. After drying, place aluminum weigh boat in desiccator until cool.
h. Recalibrate and rezero balance. Once balance is zeroed, do not touch tare knob.
i. Weigh aluminum pan and contents and record as tare and dry material.

Calculations of percent moisture from this procedure are as follows:

$$\% \text{ Moisture} = \frac{(\text{wet soil + tare}) - (\text{dry soil + tare})}{\text{wet soil - tare}} \times 100$$

To determine the number of dry grams equivalent to a known amount of wet grams the percent moisture must be used as follows:

$$\#\text{g dry soil} = \#\text{g wet soil} \times \left(1 - \frac{\% \text{ moisture}}{100}\right)$$

3.3.6 Documentation. Record explicitly the details of subsampling, compositing, and comminuting, including:

a. Sample reduction method, if used, including weight of initial sample and weight(s) of subsample(s)
b. State of sample (partially thawed, room temperature, etc.)
c. Duration of comminuting, if a machine is used
d. Equipment used

4. Reagents for Extraction, Purification, and Analysis

4.1 Requirements. Reagents must be sufficiently pure and free from contaminants to produce efficient extraction of residues and to preclude interference with analysis. Reagent or technical grade solvents may be used in most cases after redistillation in all-glass apparatus. Analytical and "pesticide" grade solvents may need to be redistilled if the method of analysis is gas chromatography with an electron capture detector (GC/EC) and/or there is any circumstance making their purity suspect. See attachment 3 for distillation of solvents. Use the grade of solvent specified in a method, as grades are often detector-specific (for example, "HPLC" grade solvent is free from UV-absorbing contaminants, whereas "pesticide" grade is free of inclusions that would interfere with an EC detector).

MRID 41659901

HET STUDY NO.: 51434
CYANAZINE IN SOIL
E.I. DU PONT DE NEMOURS AND COMPANY, INC.

START DATE: 8/10/89
END DATE: 4/25/90

CONDUCT OF STUDY

Method

This is a modified method of du Pont Study No. AMR-1325, "Method for the Determination of Cyanazine and its Metabolites in Soil". An ion-exchange cleanup has been incorporated into the original method to remove interference problems for metabolite CH882 from the methanol/water extraction. The compounds cyanazine, BV424, and BV453 cannot be determined after the methanol/water ion-exchange cleanup.

I. Methanol/water extraction

A. Weigh 5 g of homogenized soil sample into a 250 mL polypropylene centrifuge bottle.

B. Add 50 mL of 50:50 (v/v) methanol/deionized water to the centrifuge bottle. Subject the sample to sonic disruption at 300W for 4 minutes.

C. Centrifuge the sample at 10000 rpm for 10 min.

D. Decant the supernatant into a 500 mL f.b. rotary evaporator flask.

E. Repeat steps B-D, two more times, combining the extracts in the 500 mL flask.

F. Save the remaining soil solids, for the methanol/0.1 N NaOH extraction.

G. On the rotary evaporator reduce the extract volume to approximately 20 mL (50°C water bath).

H. Quantitatively transfer the concentrated extract to a 40 mL centrifuge tube.

I. Use about 5 mL of deionized water to rinse the 500 mL f.b. flask. Combine the wash with the extract in the 40 mL centrifuge tube.

J. Centrifuge at 10000 rpm for 10 min.

K. Decant the supernatant back into the 500 mL f.b. flask.

L. Reduce the volume to 2-3 mL on the cold finger evaporator (dry ice and acetone temperature).

M. Transfer the extract to a 10 mL culture tube (silanized), and combine with a 2-3 mL deionized water rinse of the 500 mL f.b. flask.

N. On the N-EVAP, reduce the volume to less than 1 mL, using a water bath at 60°C.

O. Vortex the concentrate in the culture tube, and transfer it with a pipet to a 1 mL volumetric flask. Rinse the culture tube with deionized water, vortex, and combine with the extract in the 1 mL flask.

P. Bring the volume of the extract to 1 mL with water, then invert 20 times to mix the extract.

Q. Transfer the extract to a 1.5 mL Eppendorf polyethylene centrifuge tube, and centrifuge on the Eppendorf model 5414 centrifuge for 5 minutes.

R. Transfer the supernatant to a 1 mL polypropylene insert in a WATERS 4 mL HPLC vial, and cap the vial. Save the 1.5 mL Eppendorf polyethylene centrifuge tube for the methanol/water ion-exchange cleanup.

S. If samples are not immediately analyzed, they should be stored in refrigerator at 4°C.

T. Sample must be saved after instrument analysis for the ion-exchange cleanup.

II. Methanol/Water Ion-Exchange Cleanup:

A. From Step I.R, rinse the 1.5 mL Eppendorf polyethylene centrifuge tube twice with 1 mL of 25 mM Sodium Phosphate Buffer (pH 1.8). Save this extract in a 16X100 silanized culture tube which has been labeled with the sample I.D.

B. From Step I.T, after instrument analysis, transfer the sample from the autosample vials using pastuer pipets to the silanized culture tubes from Step II.A. Rinse the autosample vials twice with 1 mL of 25 mM Sodium Phosphate Buffer (pH 1.8) and combine the wash with the extract in the culture tube. If any dilutions were required for the methanol/water extraction, only the undiluted sample will be used for the ion-exchange cleanup.

C. Add additional 25 mM Sodium Phosphate Buffer (pH 1.8) to each culture tube for a total volume of 10 mL. The sample is now ready for the ion-exchange cleanup.

Ion-Exchange Cleanup

D. Push the two frits out of the disposable cartridges with a large, straightened paper clip. Replace one of the frits back into the bottom of the cartridge.

E. Weigh out 1 g of the cation exchange resin into a small beaker. Slurry the resin in about 10 mL of pH 1.8, 100 mM phosphate buffer, and pour into the cartridge while applying gentle vacuum through use of the vacuum manifold. Rinse any resin remaining in the beaker into the cartridge using 5 mL volumes of buffer. Do not allow the resin bed to dry out until all of the resin has been transferred into the column. Press the other frit gently onto the resin bed.

F. Pre-elute the cartridge (under vacuum) with two 5 mL volumes of 50/50 (v/v) methanol:concentrated ammonium hydroxide. Allow all of the liquid to elute from the cartridge between rinses.

G. Pre-elute the cartridge with two 5 mL volumes of pH 1.8, 100 mM phosphate buffer.

H. Repeat steps F. and G. one more time.

I. Attach a 75 mL reservoir to the top of the cartridge. Pour the aqueous sample extract obtained in step II.C. into the reservoir, and pass the sample through the column into a waste container inside the vacuum manifold at a flow rate of 2-3 mL/min.

J. Rinse the cartridge with three 5 mL portions of the pH 1.8, 100 mM phosphate buffer, followed by two 5 mL portions of water. These rinses also may drain into the waste container.

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E.I. DU PONT DE NEMOURS AND COMPANY, INC.

II. Methanol/Water Ion-Exchange Cleanup: (Continued)

K. Place a 10 mL silanized culture tube containing two drops of glycerin into the vacuum manifold, under the cartridge outlet.

L. Elute the cartridge with two 2 mL portions of 50/50 (v/v) methanol:concentrated ammonium hydroxide at a flow rate of 2-3 mL/min.

M. Evaporate the eluate collected in the centrifuge tube to "dryness" (i.e., only the glycerin remains) using the N-EVAP with the water bath set at 70°C. Bring up to the 1 mL mark with pH 1.8, 25 mM phosphate buffer, mixing thoroughly on a Vortex mixer to dissolve the glycerin and rinse down the walls of the tube.

N. If there is a precipitate, transfer the extract to 1.5 mL plastic centrifuge tubes and centrifuge 5 minutes on the Eppendorf 5414 centrifuge.

O. Transfer the concentrate to a 1 mL polypropylene, insert in a 4 mL HPLC vial, and cap the vial.

P. If samples are not immediately analyzed, they should be stored in a refrigerator at 4°C.

Q. Final calculations must include a dilution factor for metabolite CH882. The HPLC removes .1 mL of extract for every injection. An additional .1 mL must be accounted for if any samples required dilutions before the ion-exchange cleanup.

III. Methanol/0.1 N NAOH (Aqueous) Extraction

A. Use the soil sample saved from step F. on the methanol/water extraction.

B. Add 50 mL of 50:50 (v/v) methanol/0.1N NaOH (aqueous) to the soil sample.

C. Subject the sample to sonic disruption at 300w for 4 minutes.

D. Centrifuge the sample at 10000 rpm for 10 min.

E. Decant the supernatant into a 500 mL f.b. rotary evaporator flask.

F. Repeat steps 2-5, two more times, combining the extracts in the 500 mL flask.

G. On the rotary evaporator reduce the extract volume to 75 mL or less (50°C water bath).

H. Transfer the concentrate to a 250 mL beaker, and rinse the 500 mL f.b. flask with deionized water, combining the rinse with the concentrate.

I. Adjust the pH of the concentrate to 2.0 by addition of 6N HCl.

J. Transfer the concentrate to a 125 mL separatory funnel.

K. Add 50 mL ethyl acetate, shake for 1 minute, drain the aqueous lower layer into the 250 mL beaker, and discard the upper ethyl acetate layer.

L. Repeat steps 10 and 11, two more times.

M. This extract can be stored overnight under refrigeration (0°C to 6°C).

IV. Ion-Exchange Cleanup

Column preparation

A. Push the two frits out of the disposable plastic column with a large, straightened paperclip.

B. Use a glass rod to push one of the frits to the bottom of the column.

IV. Methanol/0.1 N NaOH (Aqueous) Ion-Exchange Cleanup: (Continued)

C. Slurry 1 g of cation exchange resin with 10 mL of 100 mM phosphate buffer (pH 1.8) in a small beaker, and pour the slurry into the column.

D. Using a vacuum manifold, pull the excess liquid through the resin. Be very careful not to dry the resin bed out.

E. Rinse the beaker with 5 mL more of buffer and transfer the residual resin to the column. Again, pull down the excess buffer.

F. Gently press the other frit onto the resin bed.

G. Pre-elute the cartridge under vacuum with two 5 mL volumes of 50:50 (v/v) methanol/concentrated ammonium hydroxide. Allow all of the liquid to elute from the cartridge between rinses.

H. Pre-elute the cartridge with two 5 mL volumes of pH 1.8, 100 mM phosphate buffer.

I. Repeat steps G.-H. one more time.

J. Attach a 75 mL reservoir to the top of the cartridge.

V. Sample Cleanup:

A. Pour the aqueous sample extract obtained in step III M. of the methanol/.1 N NaOH Extraction into the 75 mL reservoir.

B. Pass the sample through the column into a waste container inside the vacuum manifold at a flow rate of 3-5 mL/minute.

C. Rinse the cartridge with three 5 mL portions of the pH 1.8, 100 mM phosphate buffer, followed by two 5 mL portions of water. These rinses also may drain into the waste container.

D. Place two drops of glycerol into a 10 mL culture tube(silanized), and collect column eluate in it.

E. Elute the analytes from the column with two 2 mL portions of 50:50 (v/v) methanol/concentrated ammonium hydroxide at a flow rate of 3-5 mL/min.

F. Evaporate the eluate to "dryness" so only the glycerol remains, using the N-EVAP with a 70°C water bath.

V. Methanol/0.1 N NaOH (Aqueous) Sample Cleanup: (Continued)

G. Add about 0.5 mL pH 25 mM phosphate buffer to the sample, vortex, and pipet into a 1 mL volumetric flask. Add about 5-10 drops more of buffer, vortex, and combine this rinse with the first. Bring the volume to 1 mL with buffer.

H. If there is a precipitate, transfer the extract to 1.5 mL plastic centrifuge tubes and centrifuge 5 minutes on the Eppendorf 5414 centrifuge.

I. Transfer the concentrate to a 1 mL polypropylene insert in a 4 mL HPLC vial, and cap the vial.

J. If samples are not immediately analyzed, they should be stored in a refrigerator at 4°C.

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HPLC ANALYSIS METHANOL/WATER

Instrumentation:

Waters Automated Gradient Controller Model 680

Waters UV Detector Model 484

Waters Column Heater Model TCM

Waters 501 HPLC Pump (2 ea)

Integrator Spectra-Physics 4270 or Hewlett Packard HP 3396A

Waters WISP 712 Autosampler

HPLC Conditions:

Column:	Partisil ODS-3, 5 µ, 25 cm X 4.6 mm I.D., (Alltech Cat. No. 4238001)
Guard Column:	Partisil ODS-3, 10 µ, packing material (Alltech Cat. No. 4128010)
Column Temperature:	30°C
Flow Rate:	1 mL/min.
Wavelength:	235
Back Pressure:	1800 p.s.i. (Initial Conditions, time 0)

Gradient Conditions:

	Mobile Phase		
Time	% A	% B	Curve
0	20	80	*
20	60	40	6
30	60	40	6
32	100	0	6
38	100	0	6
40	20	80	6

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E.I. DU PONT DE NEMOURS AND COMPANY, INC.

HPLC Analysis: (Continued)

Mobile Phase A:

75% Methanol
25% 4 mM Heptanesulfonic Acid, Sodium
40 mM Sodium Phosphate Monobasic
pH = 3.00

Mobile Phase B:

1 mM Heptanesulfonic Acid
10 mM Sodium Phosphate Monobasic
pH = 3.00

All samples will be stored in HET freezers for sixty (60) days after completion of the final report. After that time, samples will either be returned or disposed of unless otherwise instructed in writing.

HET STUDY NO.: 51434
CYANAZINE IN SOIL
E.I. DU PONT DE NEMOURS AND COMPANY, INC.

HPLC ANALYSIS FOR METHANOL/WATER ION-EXCHANGE CLEANUP

Instrumentation:

Waters Automated Gradient Controller Model 680

Waters UV Detector Model 484

Waters Column Heater Model TCM

Waters 501 HPLC Pump (2 ea)

Integrator Spectra-Physics 4270 or Hewlett Packard HP 3396A

Waters WISP 712 Autosampler

HPLC Conditions:

Column:	Partisil ODS-3, 5 µ, 25 cm X 4.6 mm I.D., (Alltech Cat. No. 4238001)
Guard Column:	Partisil ODS-3, 10 µ, packing material (Alltech Cat. No. 4128010)
Column Temperature:	30°C
Flow Rate:	1 mL/min.
Wavelength:	235
Back Pressure:	1800 p.s.i. (Initial Conditions, time 0)

Gradient Conditions:

	Mobile Phase		
Time	% A	% B	Curve
0	10	90	*
30	50	50	3
32	100	0	6
45	100	0	6
47	10	90	6

HET STUDY NO.: 51434
CYANAZINE IN SOIL
E.I. DU PONT DE NEMOURS AND COMPANY, INC.

HPLC Analysis: (Continued)

Mobile Phase A:

75% Methanol
25% 20 mM Heptanesulfonic Acid, Sodium
80 mM Sodium Phosphate Monobasic
pH = 3.00

Mobile Phase B:

5 mM Heptanesulfonic Acid
20 mM Sodium Phosphate Monobasic
pH = 3.00

All samples will be stored in HET freezers for sixty (60) days after completion of the final report. After that time, samples will either be returned or disposed of unless otherwise instructed in writing.

HET STUDY NO.: 51434
CYANAZINE IN SOIL
E.I. DU PONT DE NEMOURS AND COMPANY, INC.

HPLC ANALYSIS FOR METHANOL/.1 N NAOH

Instrumentation:

Waters Automated Gradient Controller Model 680

Waters UV Detector Model 484

Waters Column Heater Model TCM

Waters 501 HPLC Pump (2 ea)

Integrator Spectra-Physics 4270 or Hewlett Packard HP 3396A

Waters WISP 712 Autosampler

HPLC Conditions:

Column:	Partisil ODS-3, 5 µ, 25 cm X 4.6 mm I.D., (Alltech Cat. No. 4238001)
Guard Column:	Partisil ODS-3, 10 µ, packing material (Alltech Cat. No. 4128010)
Column Temperature:	30°C
Flow Rate:	1 mL/min.
Wavelength:	235
Back Pressure:	1800 p.s.i. (Initial Conditions, time 0)

Gradient Conditions:

	Mobile Phase		
Time	% A	% B	Curve
0	10	90	*
30	60	40	6
32	100	0	6
45	100	0	6
47	10	90	6

HET STUDY NO.: 51434
CYANAZINE IN SOIL
E.I. DU PONT DE NEMOURS AND COMPANY, INC.

HPLC ANALYSIS (Continued)

Mobile Phase A:

75% Methanol
25% 20 mM Heptanesulfonic Acid, Sodium
80 mM Sodium Phosphate Monobasic
pH = 3.00

Mobile Phase B:

5 mM Heptanesulfonic Acid
20 mM Sodium Phosphate Monobasic
pH = 3.00

All samples will be stored in HET freezers for sixty (60) days after completion of the final report. After that time, samples will either be returned or disposed of unless otherwise instructed in writing.

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STOCK STANDARDS

Name of Standard: BV424
% Purity 98.0% Ref. No. WL-020196
CODE 2-1-0-0

Name of Standard: CH882
% Purity 95.0% Ref. No. SD-031224
CODE 2-1-0-0

Name of Standard: CH881
% Purity 95.0% Ref. No. SD-031223
CODE 4-1-0-0

Name of Standard: BV453
% Purity 99.0% Ref. No. WL-020258
CODE 1-3-0-0

Name of Standard: Cyanazine
% Purity 98.0% Ref. No. SD-015418
CODE 13-1-0-0

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CALCULATIONS

ppm added = µg added/sample weight (g)

response factor (RF) = peak area or height/µg injected

µg injected = concentration of standard (µg/mL) X volume injected (mL)

$$\text{µg found} = \frac{\text{final extract volume (mL) X peak area or height}}{\text{Ave. RF X Injection Volume (mL)}}$$

ppm found = µg found/sample weight (g)

% recovery = ppm found/ppm added X 100

$$\text{\% Moisture} = \frac{\text{wet soil wt. (g) - dry soil wt. (g)}}{\text{dry soil wt. (g)}} \text{ X } 100$$

$$\text{Found Dry Wt.} = \frac{\text{Found ppm}}{1-\frac{(\text{\% Moisture})}{100}}$$

$$\text{Average Response Factor (Ave. RF)} = \frac{\text{summation of RF from standard curve}}{\text{X number of points in standard curve}}$$

HET STUDY NO.: 51434
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TABLE OF DEVIATIONS FROM DU PONT METHOD AMR-1325-88

I. Methanol/Water Extraction

A. The centrifuge was run for 10 min at 10000 rpm for step 3 and 7 of the Dupont Method.

B. The use of a cold finger evaporator was used after the initial evaporation of methanol from the samples, for step 6 of the Dupont Method.

C. The centrifuge was run for 10 min at 10000 rpm for step 7 of the procedure, and the cold finger evaporator was used instead of the rotary evaporator.

D. For step 8, solutions were transferred to a silanized culture tube (16X100).

E. The final extracts of step 10 were centrifuged with an Eppendorf centrifuge to eliminate suspended solids.

F. Ion-exchange cleanup is run after instrument analysis for metabolite CH882. (See Conduct of Study)

II. Methanol/.1 N NaOH (Aqueous) Extraction

A. The centrifuge was run for 10 min at 10000 rpm for step 12 of the Dupont Method.

B. For step 16 of Dupont Method AMR-1325-88, 6N HCl was used to pH extracts.

C. A 125 mL separatory funnel was used for step 17 of the Dupont Method.

D. The upper ethyl acetate layer was not aspirated from the top of the aqueous layer, but poured from the separatory funnel after draining the lower aqueous layer from the funnel.

E. A straightened paper clip was used to remove the frits from the plastic column and not a pipet, which easily breaks.

F. Silanized culture tubes (16X100) were used to collect extract in vacuum manifold for step 27.

G. The final extracts of step 29 of the Dupont Method were centrifuged with an Eppendorf centrifuge to eliminate suspended solids, if needed.

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E.I. DU PONT DE NEMOURS AND COMPANY, INC.

Table of Deviations (Continued):

III. HPLC Analysis

Ion pairing chromatography using Heptanesulfonic acid was utilized for instrument analysis. Details of conditions can be found in the Conduct of Study.

*Deviations from Method AMR-1325-88 were discussed with Chuck Powley of Du Pont. (1/90 - 4/90)