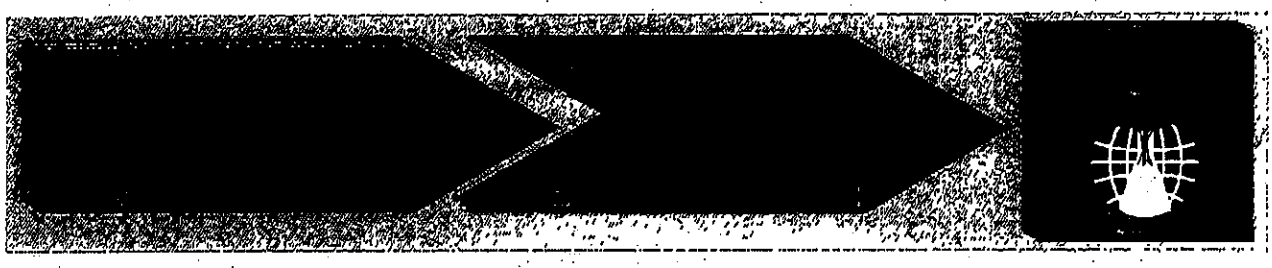

E. I. du Pont de Nemours and Company
DuPont Agricultural Products

Method No. E9650.220.01.BE
First Revision: January 30, 1995

2-BENZIMIDAZOLE CARBAMIC ACID, METHYL ESTER (IN-E965) (MBC)

Determination of Phenazines (DAP and AHP) by Reversed-Phase Liquid Chromatography (RPLC) with Fluorescence Detection

I. Principle

A portion of MBC is dissolved in an aqueous hydrochloric acid solution and precipitated with saturated sodium chloride. The filtrate is analyzed for 2,3-diaminophenazine (DAP) and 2-amino-3-hydroxyphenazine (AHP) by reversed-phase liquid chromatography. The eluted compounds pass through a post-column reagent delivery module and mix with a basic buffer-solution. The compounds are then detected with a fluorescence detector. The detector response for the sample is compared and quantitated against the response of a standard solution of phenazines.

II. Applicability

This method is applicable to the determination of DAP and AHP in MBC technical in the range of 0.1 to 5 ppm.

III. Limitations

None

IV. Sensitivity, Precision and Accuracy

A. Sensitivity

The detection limit for DAP and AHP is 50 ppb (0.05 ppm) based on signal-to-noise ratio.

B. Precision

1. Single Operator

The average analysis ($\bar{X}$), standard deviation (s) and 95% confidence limits (95% CL) established for the single operator precision of the method were as follows:

B. Precision (Cont.)

1. Single Operator

	$\bar{X}$	s	95% CL	%RSD
DAP, ppm	2.30	0.155	± 0.40	6.8
AHP, ppm	0.68	0.142	± 0.36	21

The above data were calculated from six replicate analyses of one sample performed by one technician over a period of one day.

2. Multiple Operator

Not currently available.

C. Accuracy

A spiking and recovery study was carried out by spiking an MBC technical sample at two different levels. The average recovery was 90% for DAP and 115% for AHP.

Component	Baseload ppm	ppm added	Actual ppm	Recovery %
DAP	2.10	0.685	2.76	96.4
AHP	0.45	0.52	1.12	128.8
DAP	2.10	1.37	3.25	83.9
AHP	0.45	1.04	1.43	94.2

NOTE: Linearity

The linearity of DAP and AHP was determined by spiking four different levels into MBC. The results of this study show DAP is linear up to approx 5 ppm and AHP to approx. 4 ppm (see Appendix 2).

V. Special Apparatus (Equivalent apparatus may be substituted)

1. High performance liquid chromatograph, such as the following modular system:

- Eluent pump (Beckman Model 110A)
- Buffer (post column) pump (Applied BioSystems Model 400A)
- Fluorescence Detector (McPherson Model FL-749) equipped with a high sensitivity accessory (HSA)
- Column, 150 x 4.6 mm Inertsil C8 (MetaChem)
- Hewlett-Packard 1050 Autosampler
- Hewlett-Packard 3350 Lab Automation System (LAS) or equivalent computing system

V. Special Apparatus (Equivalent apparatus may be substituted) (Cont.)

2. Solvent filters, Cat. No. XX10 047 00, Millipore Corporation.

3. Filter Discs, for organic solvents, Cat. No. FHUP-047 00, Millipore Corporation.

4. Filter flask and funnel, Cat. No. KT93825-47 and KT93840-4035, VWR Scientific.

5. Sample filters, 0.45 µm, PTFE, Cat. No. 44525-PC, Scientific Resource Inc., (SRI), PO Box 1290, Eatontown, NJ 07724, 1-800-637-7948.

6. Burrell wrist action shaker, Cat. No. 57040-027, VWR Scientific.

7. Repipet, 10 mL, Cat. No. 13-687-49N, Fisher Scientific.

VI. Reagents (Reagent grade except as noted)

1. Triethylamine (TEA), 99%, Cat. No. 13,206-3, Aldrich Chemical Co.

2. Acetonitrile (CH_3CN), HPLC grade, Cat. No. A998SK-4, Fisher Scientific.

3. Phosphoric acid (H_3PO_4), HPLC grade, 85%, Cat. No. A-260-500, Fisher Scientific.

4. Hydrochloric acid (HCl), concentrated, ACS grade, 12 N, Cat. No. A-144S-212, Fisher Scientific.

5. HCl, 1.0 N:

   Add (grad) 83 mL of concentrate HCl to 917 mL of water. Label and date. The solution is stable for one year.

6. Ascorbic Acid, "Baker Analyzed", Cat. No. B581-07, J. T. Baker.

7. Sodium Chloride (NaCl), Cat. No. S-271, Fisher Scientific.

8. Water, ASTM Type II.

9. 2,3-diaminophenazine (DAP), reference standard, IN-M959, DuPont Agricultural Products, Wilmington.

10. 2-amino-3-hydroxyphenazine (AHP), reference standard, IN-F2703, DuPont Agricultural Products, Wilmington.

11. Sodium acetate trihydrate, "Baker Analyzed", Cat. No. 3460-11, J. T. Baker.

VI. Reagents (Cont.)

12. Eluent (TEA/CH_3CN):

a. Add (grad) 1700 mL of water to a 2000-mL beaker.

b. While stirring the solution, add (syringe) 0.20 mL (200 µL) of TEA to the beaker. Keep the tip of the syringe below the surface of the solution during the addition process.

c. Calibrate a pH meter using pH 4 and pH 7.

d. Adjust the pH to 3.75 using H_3PO_4.

e. While stirring the solution, add (grad) 300 mL of CH_3CN.

f. Filter (0.45 µm) the solution quickly so the CH_3CN and the TEA does not evaporate.

g. Label the eluent, date, and store in a tightly capped bottle. The solution is stable for at least three months.

The pH and/or CH_3CN concentration may be adjusted to optimize peak separation.

13. Acetate buffer:

a. Dissolve 27.2 g ± 0.05 of sodium acetate trihydrate in 1000 mL of water.

b. Adjust the pH to 5.0 with concentrated HCl.

c. Filter (0.45 µm) the solution, label, and date. The solution is stable for at least three months.

14. Sample Solvent:

a. Weigh (to the nearest 0.01 g) 1.0 g of ascorbic acid into a 200-mL volumetric flask.

b. Dilute to volume with 1.0 N HCl.

c. Transfer to a repipet bottle and label. Prepare this solution daily. The solution is stable for 24 hours.

15. NaCl, saturated:

a. Add approx 500 g of NaCl (or enough to saturate the solution) to a 1000-mL bottle.

b. Make to volume with water.

c. Stir for at least two hours.

VI. Reagents (Cont.)

16. DAP Stock Solution (approx 20 µg/mL):

    a. Weigh (to the nearest 0.0001 g) 0.01 g of DAP reference standard into a 500-mL volumetric flask.

    b. Make to volume with CH_3CN.

    c. Ultrasonicate the solution for at least 5 minutes.

    d. Label the flask "DAP Stock" and include the µg/mL (corrected for purity) and the date.

    e. Store the solution in an amber bottle and in a dark place. The solution is stable for at least four months.

17. AHP Stock Solution (approx 100 µg/mL)

    a. Weigh (to the nearest 0.0001 g) 0.01 g of AHP reference standard into a 100-mL volumetric flask.

    b. Add (50 mL graduate) 30 mL of CH_3CN and 30 mL of water to the flask.

    c. Add (pipet) 2 mL of concentrate HCl to the flask.

    d. Dilute to volume with water.

    e. Ultrasonicate to dissolve.

    f. Label the flask "AHP Stock", date, and record the concentration on the container (corrected for purity). The solution is stable for two months if stored in the dark.

18. 2-Benzimidazole carbamic acid, methyl ester (IN-E965) (MBC), Production sample, E. I. du Pont de Nemours and Company, Belle Plant.

VII. Special Safety Considerations

A. Product Hazards

MBC may irritate eyes, nose, throat, and skin. It may cause skin irritation and sensitization. Use ventilation that is adequate to keep airborne concentrations below exposure limits. In case of contact with skin, immediately wash with soap and water. In case of eye contact, immediately flush with water for at least 15 minutes. Report to Medical. Wear the proper protective equipment for handling MBC. For more information, consult DuPont MSDS No. DU005715 for MBC.

B. Procedure Hazards

Handle syringes with care to avoid skin puncture.

Acetonitrile is flammable and toxic. Avoid breathing vapors or skin contact.

HCl and H_3PO_4 are corrosive. Avoid eye or skin contact. Do not breath vapors or mist.

DAP and AHP are mutagenic compounds and should be handled with gloves in a well-ventilated area.

Triethylamine is flammable and corrosive. Avoid contact to eyes, skin, or clothing.

Wash thoroughly after handling any of the above compounds. Refer to the suppliers MSDS for more information.

VIII. Procedure

A. Operating Conditions

Pumps

Eluent pump: Beckman Model 110A
1.0 mL/min (TEA/CH_3CN)

Buffer pump: ABI Model 400A
0.5 mL/min (acetate buffer)

Expected retention times:

DAP	approx 4.2 min
AHP	approx 9.3 min

Detector and Power Supply

Excitation wavelength	404 nm (may vary 1-2 nm, optimize for best sensitivity)
Emission filter	550 nm
Gain	0.6
Suppression	0 (ccw)
Range	0.03-0.01
Time	5

VIII. Procedure (Cont.)

A. Operating Conditions

EXCITATION WAVELENGTH OPTIMIZATION:

1. Remove the emission filter (550 nm) from the high sensitivity accessory.
2. Set the PMT gain between 600-800 volts.
3. Rotate the excitation wavelength knob slowly, from 390 nm to 410 nm.
4. Peak for maximum energy.
5. The theoretical wavelength should be 404 nm.
6. After optimizing the excitation wavelength, install the emission filter into the high sensitivity accessory.

The system must warm up for at least 15 minutes. It is best to start the system and allow it to equilibrate before beginning sample preparation.

SYSTEM STARTUP:

1. Turn on the power supply and ignite the lamp.
2. Turn on the photometer.
3. Set the lamp power to 8 and allow it to warm up for at least fifteen minutes.
4. Adjust the GAIN so the meter reads 0.6.
5. Turn the SUPPRESSION until the meter reads 0.0-0.1.
6. Change the RANGE from 0.03 to 0.01.
7. Adjust the SUPPRESSION if necessary for the meter to read 0.1-0.2.
8. Leave the detector "ON" at all times. (See Comment 1)

B. Calibration

Prepare the working standard daily:

Add (pipet) 5.0 mL of DAP Stock standard and 1.0 mL of AHP Stock Standard to a 100-mL volumetric flask and dilute to volume with water. Mix and label the flask.

C. Sampling

Samples should be taken in clean, dry containers.

D. Sample Analysis

BLANK

1. Prepare a "blank" by weighing (to the nearest 0.0001) 1 g ± 0.005 of a retained MBC sample (known to be low in both DAP and AHP) into a 2-oz amber bottle. (See Comment 2)

2. Add (repipet) 10 mL of sample solvent and swirl until the MBC completely dissolves. (See Comment 3)

3. Add (grad) 30 mL of saturated NaCl solution.

4. Shake for 2 ± 0.5 minutes on a Burrell wrist action shaker.

5. Immediately (within 5 minutes) filter (0.45 µm) the solution into an HPLC vial for analysis.

SPIKE

6. Prepare a "spike" by weighing (to the nearest 0.0001) 1 g ± 0.005 of the same MBC used in VIII.D.1 into a 2-oz bottle.

7. Add (pipet) 2 mL of the working standard (VIII.B) into the bottle.

8. Add (repipet) 10 mL of sample solvent and swirl until the MBC completely dissolves. (See Comment 3)

9. Add (grad) 28 mL of saturated NaCl solution.

10. Shake for 2 ± 0.5 minutes on a Burrell wrist action shaker.

11. Immediately (within 5 minutes) filter (0.45 µm) the solution into an HPLC vial for analysis.

SAMPLE

12. Prepare the sample by weighing (to the nearest 0.0001) 1 g ± 0.005 of the MBC sample into a 2-oz bottle.

13. Add (repipet) 10 mL of sample solvent and swirl until the MBC completely dissolves. (See Comment 3)

14. Add (grad) 30 mL of saturated NaCl solution.

D. Sample Analysis (Cont.)

15. Shake for 2 ± 0.5 minutes on a Burrell wrist action shaker.

16. Immediately (within 5 minutes) filter (0.45 µm) the solution into an HPLC vial for analysis.

17. Load samples and standards into the autoinjector and start the analysis.

INSTRUMENT SHUTDOWN

1. Turn off the recorder and cap the pen.

2. Switch the eluent to a FLUSH solution of 75% CH_3CN and 25% H_2O.

3. Switch the post column solution to a FLUSH solution of 95% H_2O and 5% CH_3CN.

4. Flush both pumps for at least 30 minutes, then turn the pumps off.

5. Leave the detector ON.

E. Calculations

$$\text{DAP, ppm} = \frac{A \times B \times C \times D \times 10^6}{(E - F) \times G \times H \times I}$$

where,

A = area of DAP in the sample (from chromatogram)
B = grams of DAP in the stock std (approx. 0.01 g) (Step VI.16.a)
C = mL of stock solution in working standard (5 mL) (Step VIII.B)
D = mL of working standard in the spike (2 mL) (Step VIII.D.7)
E = area of DAP in the spike (from chromatogram)
F = area of DAP in the blank (from chromatogram)
G = volume of DAP stock solution (500 mL) (VI.16.a)
H = volume of DAP working standard (100 mL) (VIII.B)
I = sample weight (g) (VIII.D.12)

Simplified DAP calculation:

$$\text{DAP (ppm)} = \frac{A \times B \times 200}{(E - F) \times I}$$

E. Calculations (Cont.)

$$\text{AHP, ppm} = \frac{J \times K \times L \times D \times 10^6}{(M - N) \times O \times H \times I}$$

J = area of AHP in the sample (from chromatogram)
K = grams of AHP in the stock std (approx. 0.01 g) (Step VI.17.a)
L = mL of stock solution in working standard (1 mL) (Step VIII.B)
D = mL of working standard in the spike (2 mL) (Step VIII.D.7)
M = area of AHP in the spike (from chromatogram)
N = area of AHP in the blank (from chromatogram)
O = volume of AHP stock solution (100 mL) (VI.17.a)
H = volume of AHP working standard (100 mL) (VIII.B)
I = sample weight (g) (VIII.D.12)

Simplified AHP calculation:

$$\text{AHP, ppm} = \frac{J \times K \times 200}{(M - N) \times I}$$

Report results to the nearest whole ppm.

IX. Quality Control

Analyze a retained control sample with every set of samples and monitor the results using appropriate statistical control techniques (e.g., CUSUM, Shewhart, ANOVA, etc.). If results fall outside established limits, perform the appropriate corrective action before proceeding with sample analysis. Possible corrective actions may include, but are not limited to, the following:

1. Check instrument settings.
2. Check expiration dates on the standards, and reprepare if necessary.
3. Reprepare eluent.
4. Reanalyze the control sample in duplicate. If results are still outside the established limits, notify supervision or a member of the technical staff.

X. Comments

1. If a large number of samples are analyzed daily, leave the detector ON all the time as the detector operates better if left ON all the time.
2. The MBC sample use for the blank and the spike should have been determined to contain less than 0.1 ppm AHP and 0.5 ppm DAP per the conditions of this method.

X. Comments (Cont.)

3. Benomyl will not completely dissolve in the sample solvent.

4. For Benomyl AHP and DAP analyses, use benomyl in the blank and spiked standard calibration solution.

5. The retention times for AHP and DAP in MBC are different from that of benomyl. In the case of MBC, all the MBC is dissolved in the sample solution which has a matrix effect on the retention times of AHP and DAP.

XI. References

1. DuPont MSDS #DU005715 for MBC.

XII. Appendix

1. Benomyl Method Information
2. Linearity Data for MBC
3. MBC Blank Example Chromatogram
4. MBC Spiked Standard Example Chromatogram
5. MBC Sample Example Chromatogram
6. DAP and AHP Structures

APPENDIX 1

Benomyl Method Information

I. Safety

Benomyl technical may irritate eyes, nose throat, and skin. It may cause skin irritation and sensitization. Use ventilation that is adequate to keep airborne concentrations below exposure limits. In case of contact with skin, immediately wash with soap and water. In case of eye contact, immediately flush with water for at least 15 minutes. Report the Medical. Wear the proper protective equipment for handling Benomyl. For more information, consult DuPont MSDS No. DU003040.

II. Accuracy

A spike and recovery study was carried out by spiking a benomyl technical sample with DAP and AHP. The recovery was 98% for DAP and 113% for AHP. See DuPont Laboratory Notebook: E56564, page 55 for more information.

COMPONENT	BASELOAD	ACTUAL, ppm	ppm ADDED	RECOVERY, %
DAP	ND	1.30	1.33	98
AHP	ND	1.29	1.14	113

III. Comments

1. Benomyl will not completely dissolve in the sample solvent.

2. For Benomyl AHP and DAP analyses, use benomyl in the blank and spiked standard calibration solution.

3. The retention times for AHP and DAP in MBC are different from that of benomyl. In the case of MBC, all the MBC is dissolved in the sample solution which has a matrix effect on the retention times of AHP and DAP.

   Expected retention times (Benomyl): DAP approx 3.8 min
   AHP approx 8.5 min

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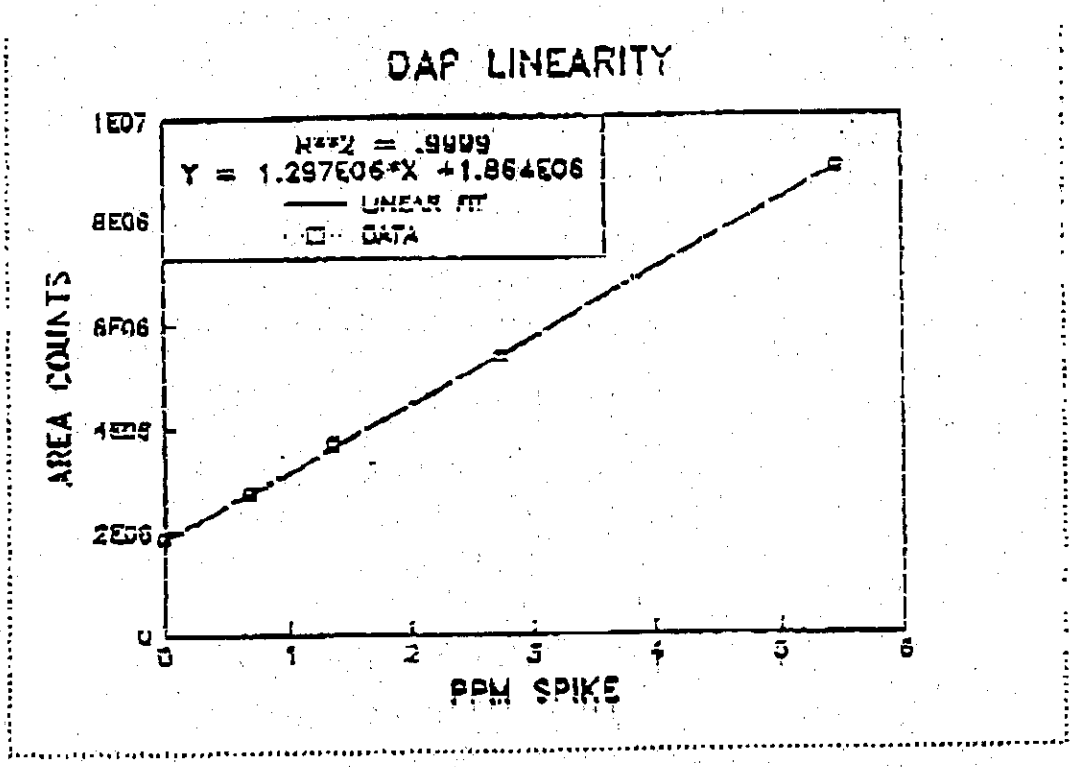
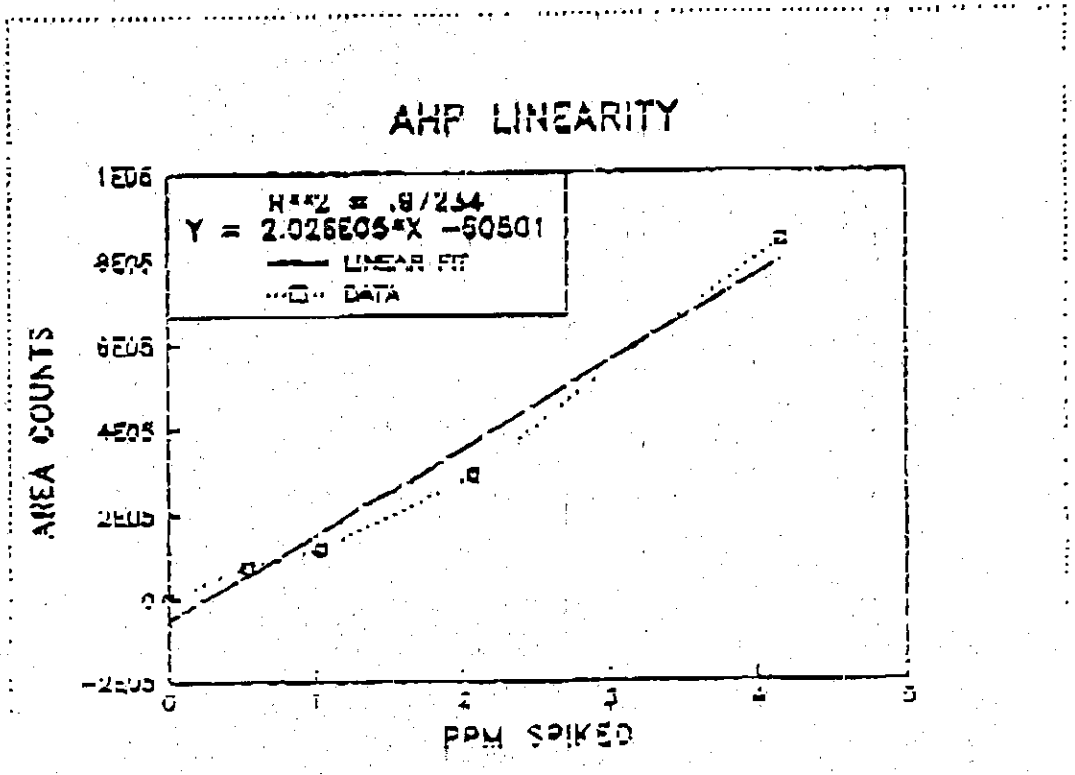
APPENDIX 2

Linearity Data for MBC





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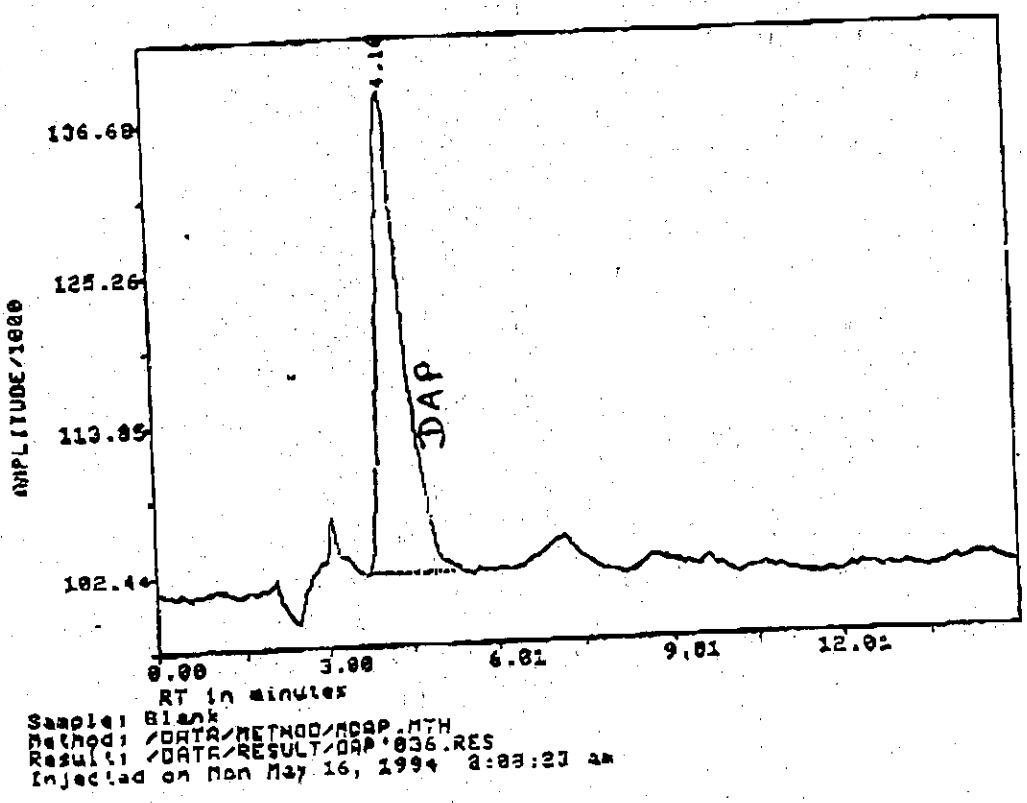
APPENDIX 3

MBC Blank Example Chromatogram



Pk#	RT	ID-tm	Peak Width	Area	Code	%AREA	Name
1	4.18	#4.19	0.0000000	2214580	FF	0.000	DAP

Total Area : 2214580 Total %AREA : 0.0

Report Time : Tue Nov 22, 1994 10:34:56 am
Method : /DATA/METHOD/MDAP.MTH
Meth Par File :
Report File : /DATA/LOOP/FORMAT/GMEDIUM_AREA.FMT

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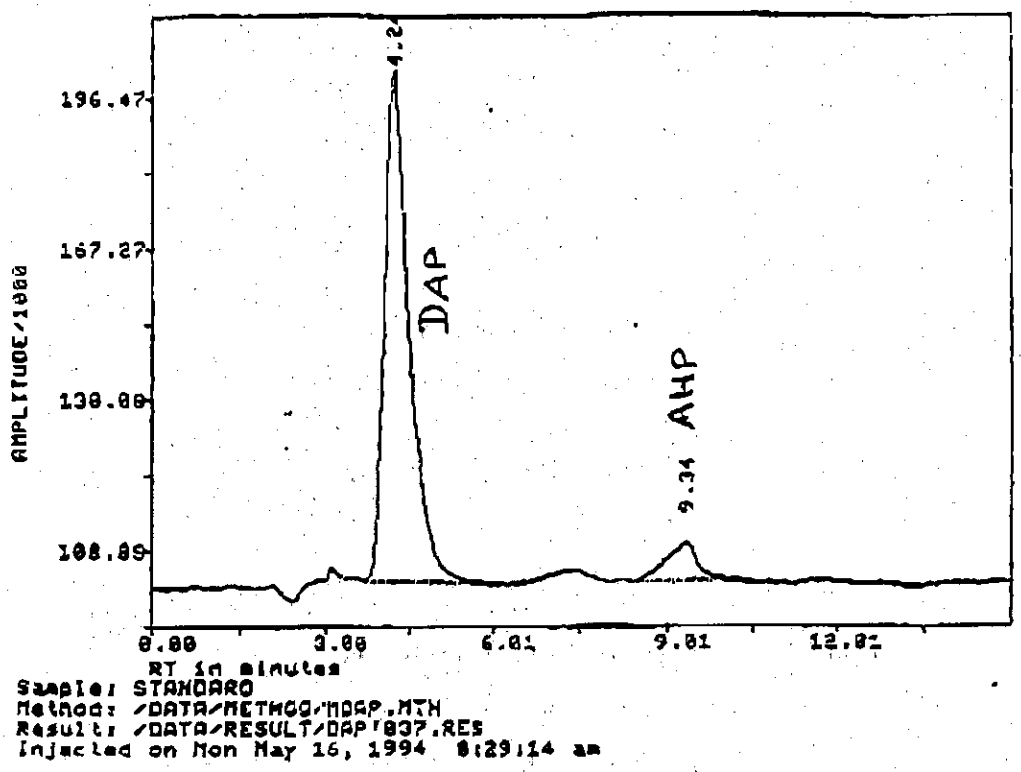
APPENDIX 4

MBC Spiked Standard Example Chromatogram



Pk#	RT	ID-tm	Peak Width	Area	Code	%AREA	Name
1	4.24	#4.20	0.0000000	5759648	FF	0.000	DAP
2	9.34	9.31	0.0000000	578800	FF	57880000.0	AHP

Total Area : 6338448 Total %AREA : 57880000.000

Report Time : Tue Nov 22, 1994 10:36:04 am
Method : /DATA/METHOD/MDAP.MTH
Meth Par File :
Report File : /DATA/LOOP/FORMAT/GMEDIUM_AREA.FMT

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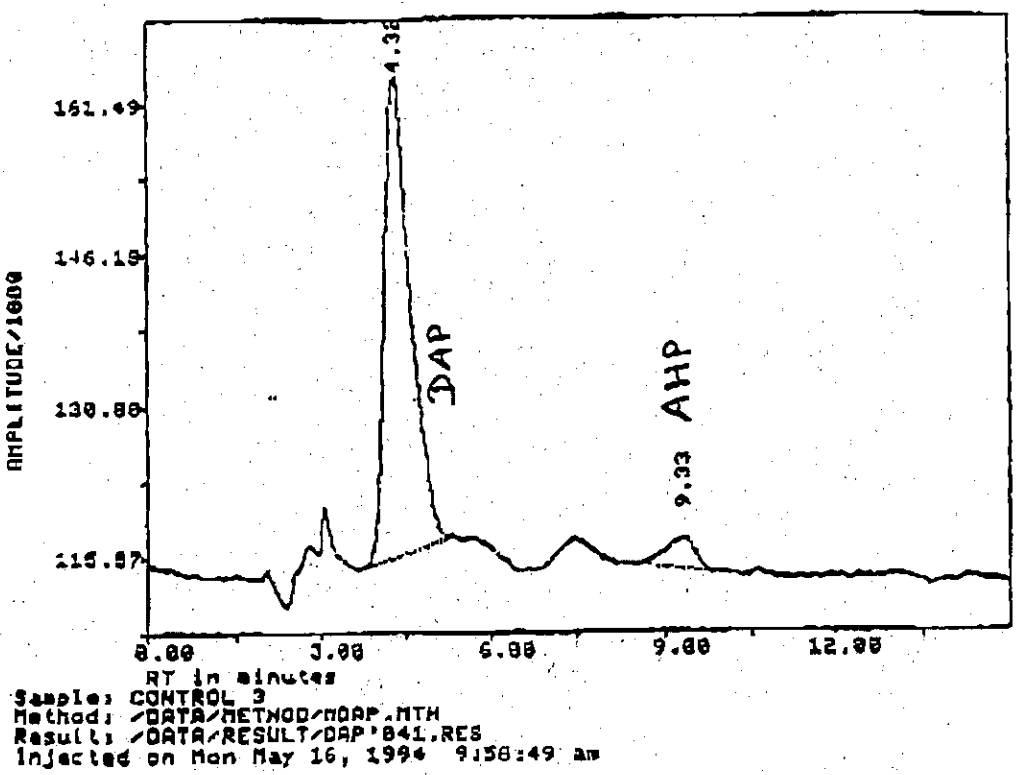
APPENDIX 5

MBC Sample Example Chromatogram



Pk#	RT	ID-tm	Peak Width	Area	Code	%AREA	Name
1	4.32	#4.24	0.0000000	2918312	FF	0.000	DAP
2	9.33	9.40	0.0000000	234100	FF	22483676.0	AHP

Total Area : 3152412 Total %AREA : 22483676.000

Report Time : Tue Nov 22, 1994 10:38:06 am
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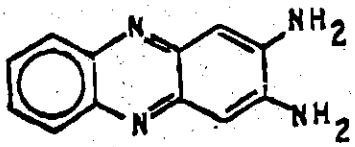
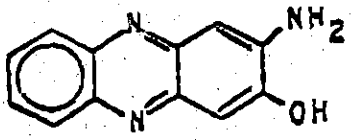
Report File : /DATA/LOOP/FORMAT/GMEDIUM_AREA.FMT

APPENDIX 6

DAP and AHP Structures



2,3-diaminophenazine



2-amino-3-hydroxyphenazine (AHP)