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EMSL-LV TSC-20

March 30,1994

ANALYTICAL METHOD DEVELOPMENT AND RESULTS FOR ß-NAPHTHYLAMINE AND 4-NITROANILINE

IN SOIL AND SEDIMENT SAMPLES

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Philadelphia, Pennsylvania

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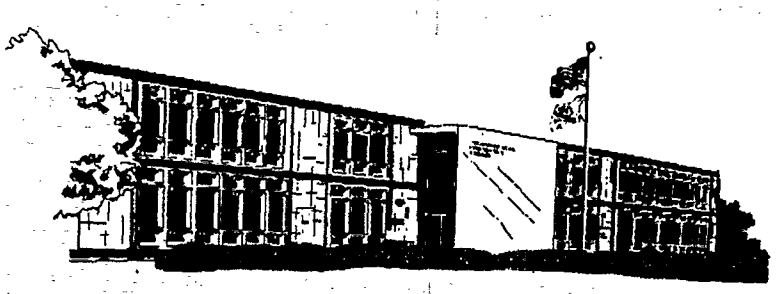

U. S. Environmental Protection Agency, Environmental Monitoring Systems Laboratory - Las Vegas

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Technology Support Project

March 30, 1994

ANALYTICAL METHOD DEVELOPMENT AND RESULTS FOR ß-NAPHTHYLAMINE AND 4-NITROANILINE IN SOIL AND SEDIMENT SAMPLES

SUPPORT FOR DRAKE CHEMICAL NPL SITE
WORK ASSIGNMENT 22410183

BY

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Contract Number 68-CO-0049

Prepared for

U.S. ENVIRONMENTAL PROTECTION AGENCY
ENVIRONMENTAL MONITORING SYSTEMS LABORATORY
OFFICE OF RESEARCH AND DEVELOPMENT
LAS VEGAS, NEVADA 89119

March 30, 1994

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INTRODUCTION

Region 3 submitted to the EMSL-LV a request for analytical support in determination of ß-naphthylamine (2-aminonaphthalene) in soils from the Drake Chemical NPL site. At this site, incineration will be performed on approximately 200,000 cubic yards of soil and sludge. The detection level of 1 ppb is desired for quality assurance purposes, and of 55 ppb for routine verification of cleanup. Additionally, 4-nitroaniline is sought as a lower-priority target analyte. This project represented an analytical challenge because standardized methodology had not been developed or validated.

Four objectives were identified by the EPA for this support project:

(1) develop an analytical procedure(s) to assure EPA that the incinerator contractor can meet the 55 ppb cleanup criterion on a daily basis, in the field, with the incinerator they propose to use. An ultimate detection limit of 1 ppb would be useful.

(2) analyze samples taken from a location on the site which was not sampled in the previous study (1987).

(3) verify the presence of ß-naphthylamine at the site above the cleanup goal level, and the presence at very high levels in certain lagoons.

(4) analyze the samples for 4-nitroaniline, as a lower priority. This compound was found on the Site in 1987.

In this report, research and testing were performed on available analytical techniques and methods. Two candidate analytical procedures were identified. These procedures were tested for the ability to attain a suitable detection limit for ß-naphthylamine and the nitroanilines, with sufficient precision and accuracy to support the Region's needs. This report summarizes the approaches used to achieve the analytical requirements, including the candidate analytical methods tested.

PHYSICAL PROPERTIES OF THE TARGET ANALYTES

The primary target analyte, ß-naphthylamine, is a white to reddish crystalline solid, mp. 111-113°C, volatile in steam and having bp. 294 or 306°C. It is a fluorescent and weakly basic compound with a pK_a of 4.07. It is soluble in hot water, alcohol, and ether, and has a density of 1.0614. Para-nitroaniline (4-nitroaniline) is a yellow powder of mp 146°C, soluble in water, alcohol, ether, benzene and methanol. It is used commercially as a dyestuff intermediate; it is weakly fluorescent, and has a pK_a of 1.0.

Under neutral conditions the target analytes are reported to dissolve in low-polarity or moderate polarity organic solvents including diethyl ether, benzene, acetonitrile, methanol, and methylene chloride. The extract can be analyzed by high-performance liquid chromatography (HPLC) coupled with mass spectrometry (MS) or other detectors, including UV-vis and fluorescence (for ß-naphthylamine). Fluorescence detection provided sensitivity and selectivity, with the desired detection limit for ß-naphthylamine (1 ppb).

METHOD DEVELOPMENT RESULTS

The major difficulty experienced was extracting the target analytes from the soil type provided as a background for the Drake Site. A brief literature search uncovered chemical reasons for the difficulty (see Appendix), and provided a background from which procedures could be developed to improve the recoverability of the analytes from the soil. In general, both fast, reversible reactions and slow, irreversible reactions of ß-naphthylamine occur with the humic materials in the soil. The irreversible reactions involve the formation of covalent bonds which chemically alter the analyte and make it integral with the humic material. Wet soil provided poorer recoveries than dry soil.

The analytical method developed in this study provided reproducible (ca. 20-25%) recoveries of the target analytes spiked onto the Drake-type background soil, and excellent recoveries on Site samples (70-99% on ß-naphthylamine and 72-96% on 4-nitroaniline, both spiked at 1 ppm). The method detection limit was in accordance with project objectives (1 ppb for ß-naphthylamine). Analysis by HPLC, with fluorescence and mass spectrometric detectors, was found to be suitable. The method used to analyze the Site samples ("Final Analytical Method") was the second candidate method tested.

ANALYTICAL RESULTS SUMMARY

The Drake Chemical Site samples were analyzed by the Final Analytical Method presented in this report.

Analytical results are presented in Table 1.

Samples were analyzed in several groups. Initial and continuing calibrations (Tables 2-6) are presented after the sample analysis data. Analytical results on Site samples are presented to two significant figures. Non-detects are labeled ND. The method detection limits were 1 ppb for ß-naphthylamine and 300 ppb (0.3 ppm) for 4-nitroaniline. Duplicate samples are labeled DUP. The 4-nitroaniline results were taken on undiluted extracts (dilution factor 1) except as noted. If an interference prevented detection and quantitation of the analyte in a sample, "Interf." is listed in the table.

Matrix spike (MS) and matrix spike duplicate (MSD) recoveries in percent are shown in the concentration columns. The matrix spike recoveries are corrected for the amount of analytes present in the samples before spiking. The spikes added a 1 ppm concentration of each analyte to the soil.

Region 3 supplied separate sample bottles labeled DUP, MS and MSD. Difficulties in sampling may have created some sample heterogeneity. It is believed that heterogeneity was a factor in DUP, MS and MSD data variability for the Region 3 generated DUP, MS, and MSD samples. Spike results were acceptable in the case of 7.12A, a sample in which the analytes were not detected before spiking. On the other hand, 11.12A and 11.12A DUP were very different in appearance and in analytical results. Percent recoveries for the MS and MSD are not shown in the table because the analytical results indicated that the four bottles probably contained effectively different samples, rather than four aliquots of a homogeneous single sample.

Several additional MS and MSD samples were generated in this laboratory, in each case using the single bottle having that sample number, supplied by the Region. These laboratory-generated MS and MSD samples were potentially more homogeneous, as they were taken from a single sample bottle. Data for these samples gave close agreement to the expected values, and indicated that the method performed well on the Site samples. Laboratory-prepared MS and MSD samples are labeled MS* and MSD* in Table 1.

Table 1. Analytical Results on Drake Chemical Site Samples				
Sample #	ß-NAP Dilution Factor	Conc. 4-NIT (ppm)	Conc. ß-NAP (ppb)	Comments
8.2A	1	ND	120	
8.6A	1:1000	Interf.	210,000	
8.12A	1:1000	Interf.	27,000	
4.2A	1:100	2.8	5000	
4.6A	1:100	0.26	820	
4.12A	1:100	2.7	990	
2.2A	1:1000	ND	88,000	
2.6A	1:100	ND	4400	
2.12A	1:100	ND	2800	
2.12A DUP	1:100	ND	2100	DUP Reg. 3 generated
9.2R1	1	ND	ND (a)	
9.2R2	1	ND	17	
9.2R3	1	ND	39	
9.2A	1	ND	53	
9.2A MS*	1:10	83%	71%	MS lab generated
9.2A MSD*	1:10	87%	70%	MSD lab generated
9.6B	1	ND	16	
9.12A	1	ND	8.2	
10.2A	1	ND	82	
10.6A	1	ND	26	
10.6A MS*	1:10	89%	86%	MS lab generated
10.6A MSD*	1:10	72%	90%	MSD lab generated
10.11A	1	2.0	82	

Table 1, contd. Analytical Results on Drake Chemical Site Samples.				
Sample #	ß-NAP Dilution Factor	Conc. 4-NIT (ppm)	Conc. ß-NAP (ppb)	Comments
10.1R1	1:10	ND	100	
10.1R2	1:10	ND	430	
10.1R3	1	ND	ND (a)	
7.2A	1	ND	1.5	
7.6A	1:1000(both analytes)	ND (b)	22,000	
7.12A	1	ND	ND	
7.12A DUP	1	ND	ND	DUP Reg. 3 generated
7.12A MS	1:10	86%	73%	MS Reg. 3 generated
7.12A MSD	1:10	94%	89%	MSD Reg. 3 generated
6.2A	1:10	ND	780	
6.6A	1:100	ND	1300	
6.12A	1:100	ND (a)	1100	
6.2R1	1	ND	200	
6.2R2	1:10	ND	1500	
6.2R3	1	ND	560	
11.2A	1	ND	8.5	
11.6A	1	ND	61	
11.12A	1:100 (both analytes)	52	12,000	more oily than DUP, MS, MSD
11.12A DUP	1:10 (both analytes)	18	1700	DUP Reg. 3 generated
11.12A MS	1:100 (both analytes)	13	2500	MS Reg. 3 generated
11.12A MSD	1:10 (both analytes)	24	2500	MSD Reg. 3 generated

Table 1, contd. Analytical Results on Drake Chemical Site Samples.

Sample #	ß-NAP Dilution Factor	Conc. 4-NIT (ppm)	Conc. ß-NAP (ppb)	Comments
12.2A	1	ND	ND	
12.6A	1:100	Interf.	7500	
12.12A	1	ND	1.6	
12.12A MS*	1:10	93%	92%	MS lab generated
12.12A MSD*	1:100	96%	99%	MSD lab generated
5.2A	1	ND	180	
5.6A	1	ND	22	
5.12A	1	ND	2.1	
3.2A	1	ND	3.9	
3.6A	1	ND	10	
3.12A	1	ND	14	
1.2A	1	ND	11	
1.6A	1	ND	180	
1.12A	1	ND	170	
1.12A DUP	1	ND	45	DUP Reg. 3 generated
1.12A MS*	1:10	92%	79%	MS lab generated
1.12A MSD*	1:10	92%	76%	MSD lab generated

ß-NAP = ß-naphthylamine; 4-NIT = 4-nitroaniline
ND = not detected; method detection limits 1 ppb for ß-NAP and 0.3 ppm for 4-NIT.
Interf. = interference prevented detection and quantitation.
(a) analyte retention time has some interference from a close-eluting peak.
(b) Sample extract cloudy and was diluted before analysis. 4-nitroaniline was not detected, but dilution degraded its detection limit in this extract by a factor of 1000.

Initial calibration results are as follows:

Table 2. Initial Calibration #1			
ß-Naphthylamine		4-Nitroaniline	
Conc. (ppb)	RF	Conc. (ppm)	RF
2.00	2.65	0.333	53.8
10.0	2.72	1.67	56.4
50.0	2.88	8.33	56.1
250	2.94	16.7	56.3
417	3.01	41.7	56.5
Avg.RF = 2.84, %RSD = 5.34		Avg.RF = 55.8, %RSD = 2.00	

Table 3. Initial Calibration #2			
ß-Naphthylamine		4-Nitroaniline	
Conc. (ppb)	RF	Conc. (ppm)	RF
1.67	1.72	0.333	52.2
10.0	2.35	1.67	53.7
50.0	2.64	8.33	57.3
250	2.75	16.7	56.3
667	2.82	41.7	56.1
Avg.RF = 2.46, %RSD = 18.3%		Avg.RF = 55.1., %RSD = 3.8%	

Continuing calibrations were performed with the solution containing 50.0 ppb ß-naphthylamine and 8.33 ppm 4-nitroaniline.

Table 4. Continuing Calibrations associated with Initial Calibration #1

ß-Naphthylamine		4-Nitroaniline	
RF	%Diff.	RF	%Diff.
2.79	1.76%	56.6	1.41%
2.76	2.82%	56.5	1.25%
2.69	5.28%	57.1	2.33%

Table 5. Continuing Calibrations associated with Initial Calibration #2

ß-Naphthylamine		4-Nitroaniline	
RF	%Diff.	RF	%Diff.
2.75	11.9%	56.5	2.5%
2.63	6.9%	56.9	3.3%
2.50	1.8%	56.8	3.1%
2.72	10.4%	54.7	0.7%
2.52	2.3%	56.1	1.7%
2.39	2.9%	57.1	3.6%
2.70	9.8%	57.6	4.5%
2.31	6.1%	57.5	4.3%
2.78	13.0%	57.4	4.1%
2.88	17.1%	57.0	3.4%
2.67	8.5%	58.3	5.8%
2.36	4.1%	60.2	9.2%
2.32	5.7%	55.3	3.3%
2.24	8.9%	57.8	1.9%

Table 6. Other Calibrations, for 4-Nitroaniline only			
Initial Calibration		Continuing Calibrations (at 0.250 ppm)	
Conc. (ppm)	RF	RF	% Diff.
0.250	60.8	69.0	17.3%
1.00	55.2	53.8	8.6%
10.0	60.3	56.2	4.4%
Initial Calibration Avg. RF = 58.8, 5.3% RSD		67.3	14.4%
		58.5	0.54%
		54.2	7.9%
		58.5	0.54%
		59.2	0.68%
		55.2	6.2%

METHOD DEVELOPMENT STRATEGY

The method development included two general areas: (1) sample extraction, and (2) sample analysis. A background sample was supplied by Region 3. Region 3 considered this sample to be the most representative available background soil for the Drake site soils. Aliquots of this background sample were used to develop and evaluate the analytical method. Analytical results indicated that the background soil provided significantly poorer recoveries of the analytes, and the background soil lacked potential analytical interferences that were found in some Site samples.

It should be noted that the matrix spike results could not accurately mimic every binding mechanism for the analytes to the matrix. Available literature reported that the analyte could react with the matrix, forming covalent bonds to humic materials in the soil. Some of these reactions are slow but essentially irreversible. Such transformation products or "metabolites" would not be extracted by solvation processes. The standard procedure for matrix spikes is to add a relatively high concentration of the spike and extract shortly thereafter. This procedure is reasonable to mimic fast electrostatic binding and some adsorption or absorption processes. It does not accurately reflect the covalent reactions that occur slowly over long periods of time, as was the case with the target analytes at the Drake Site. Therefore, these analytical results indicate a minimum probable concentration that is present (or was present before transformation) at the Drake Site.

Sample Extraction

Two sonication-based sample extraction methods were evaluated: an aqueous acid extraction at pH 1.5 and neutral SW-846 Method 3550 extractions with methanol, acetonitrile, and methylene chloride. Acidic sample extracts were neutralized and back-extracted into methylene chloride.

Soxhlet and shaker extractions were also evaluated. Soxhlet extraction is generally considered to be a rigorous extraction procedure that can yield the highest recoveries for most analytes. Shaker extraction avoids the introduction of heat, even the potential for spot-heating which could occur with sonication.

Analysis by HPLC-UV, after solvent exchange into acetonitrile (ACN) indicated that the background soil extracts did not contain interferences. Therefore, extract cleanup methodology was not developed.

HPLC with MS Detection

The HPLC/MS studies were performed to verify the lack of interferences, using a Hewlett-Packard system consisting of a 1090L liquid chromatograph, thermospray interface, and 5988A mass spectrometer. For thermospray sensitivity enhancement, 0.75 mL/min 0.1 M NH_4OAc was added post-column. Preliminary experiments showed that 4-nitroaniline had a retention time of 8.2 min, with an estimated instrument detection limit of 200 pg, monitoring m/z 156 for the $[M+NH_4]^+$ ion. The primary target analyte, ß-naphthylamine, had a retention time of 15.0 min and an instrument detection limit of 200 pg, monitoring m/z 144 for the $[M+H]^+$ ion.

The 200 pg instrument detection limit indicated that for a 2 g sample and 100% analyte recovery through the Final Analytical Method, a 35 ppb detection limit would be obtained.

Quantitations were not performed by HPLC/MS; this technique was used only to verify the lack of coeluting compounds at the retention times of the target analytes.

HPLC with Fluorescence and UV Detection

Using a Supelco LC18-DB column, 25 cm x 2.1 mm, with 2 cm x 2.1 mm HP C-18 guard column, a flow rate of 0.25 mL/min and an oven temperature of 37.5°C, the following instrument detection limits were obtained: 1 ng for either analyte at 380 nm (UV); 6.0 pg for ß-naphthylamine using fluorescence (excitation wavelength, 232 nm; emission 410 nm). Diode array UV detection was at 380 nm for 4-nitroaniline and 234 nm for ß-naphthylamine.

Fluorescence detection provided adequate selectivity against analytical interferences. UV detection was insufficiently selective. Interferences were not noted in the background soil examined in this study. Site soils, however, containing numerous interferences to UV detection, and also contained other fluorescing compounds. The combination of HPLC separation and the more selective fluorescence detection were adequate to remove interferences for ß-naphthylamine. Potentially interfering compounds were not observed for 4-nitroaniline. The linear range of the fluorescence detector corresponded to extract concentrations of approximately 1 ppb to 500 ppb for ß-naphthylamine. The HPLC-fluorescence instrument was calibrated over the range 2 to 417 ppb. The detection limit for 4-nitroaniline was 0.3 ppm (300 ppb). The calibration for 4-nitroaniline was 0.333 to 41.7 ppm. All analytical quantitations presented in this report were based upon HPLC/fluorescence results.

QUALITY ASSURANCE

QA objectives for this project were based upon method performance on background soil. A single-laboratory test of the proposed method was performed to verify that these objectives could be met routinely. For the Site samples, recovery values were obtained on matrix spike samples. For quality assurance, blanks, matrix spikes, and matrix spike duplicate samples were analyzed. A method blank and a laboratory control sample (LCS) using a sand matrix were analyzed with each ten Site samples. LCS recoveries were 78-100%. Instrument blanks, determined after approximately every 10 analyses, were all ND. Continuing calibrations were run before and after approximately every 20 samples analyzed. Method blank results were ND to 2.3 ppb.

The QA objectives and goals were met or exceeded in this project. The detection limit for ß-naphthylamine and matrix spike recoveries were two method features that were poorly addressed by existing technologies. The Region located methods having detection limits for ß-naphthylamine of 330-660 ppb. These limits were inadequate for Region 3 needs at this Site. Poor analyte recovery could seriously degrade both the detection limit and quantitation accuracy. In this technical support project, recoveries over 70% and a method detection limit of 1 ppb for ß-naphthylamine were obtained.

Analytical Acceptance Criteria	
Required method detection limit for ß-naphthylamine	55 ppb
Ideal method detection limit	1 ppb
Initial calibration linearity	RSD $\leq$ 25%
Continuing calibration RF	% Difference $\leq$ 25%
Required matrix spike duplicate (MSD) sample results	$\pm$ 50%
Ideal MSD sample results	$\pm$ 20%
Blank samples	analytes ND at required MDL
Required recovery of spiked analytes	15%-125%
Ideal recoveries of spiked analytes	50%-110%
Extract cleanup quality	quantitation unaffected within required duplicate precision level ($\pm$ 50%)

EXPERIMENTAL METHOD DEVELOPMENT RESULTS

Preliminary Testing

SW-846 method 3550 (sonication extraction twice) was performed on 2 g soil samples for initial testing. The table below summarizes results for 4-nitroaniline (4-NIT) and ß-naphthylamine (ß-NAP). In the table below, Soil (1) was a blank dry soil; soil (2) was wet background soil. Soil samples were individually spiked with analytes to arrive at soil concentrations of 20 ppm or 10 ppm. Solvent abbreviations are ACN for acetonitrile, MeOH for methanol. These tests indicated several points: (1) water in the extraction can be a problem, possibly due to the solubility of ß-naphthylamine in water and volatility in steam; (2) recoverability of ß-naphthylamine is poor from the soil matrix of interest, although good recoveries can be obtained from sand; (3) addition of water and pH adjustments do not enhance recoveries. The water sample was 10 mL, spiked to 10 ppm, adjusted to pH 1.5, neutralized, and extracted by solid phase extraction. Note that these preliminary, investigative experiments were each performed once, so the numbers do not include a measure of reproducibility. These experiments were selected to provide a rapid survey of potential method features.

Preliminary Tests of Method Features

matrix	Spiked Soil conc. (ppm)	Solvent	% Recovery 4-NIT	β-NAP
sand	20	ACN	47	18
soil (1)	20	ACN	42	19
soil (1)	20	H_2O, pH1 sep. funnel	10	0
sand	20	CH_2Cl_2	65	57
sand	20	CH_2Cl_2 pH9.5	62	56
sand	20	CH_2Cl_2/acetone	31	10
sand	20	H_2O/MeOH pH2	43	10
sand	20	MeOH	85	56
sand	20	MeOH pH 8.5	97	90
soil (2)	10	MeOH	50	15
soil (2)	10	MeOH pH 8.5	59	21
soil (2)	10	MeOH pH >10	16	4
soil (2)	10	CH_2Cl_2 basic	36	20
soil (2)	10	CH_2Cl_2/acetone basic	26	10
soil (2)	10	H_2O pH 1.5/ SPE	0	0
water	10	H_2O pH 1.5/ SPE	0	0

Further Testing to Investigate Specific Issues

The following tests were performed on the near-Site background soil. An aliquot of the soil was determined to be 20% moisture by weight difference upon drying in an oven. Each experiment was performed once, to provide qualitative guidance regarding desirable method features. The analytes were ß-naphthylamine (stock solution at 1000 µg/mL) and 4-nitroaniline (stock solution at 1000 µg/mL); 40 µL of stock solutions spiked onto the matrix, unless otherwise specified below. Extracts were solvent exchanged into acetonitrile, and diluted by adding 250 µL extract to 750 µL of 0.01M acetate buffer solution. Analyses were performed by HPLC with UV detection. Specific results and raw data are available upon request. The following result summaries are intended to provide some guidance about specific experimental variables that can be selected and controlled. Results from these tests assisted in the selection of suitable method features.

The effect of solvent was tested on 2 g soil samples spiked with analytes, and 2 g sodium sulfate drying agent. Ten mL solvent was used, with triple sonication, Kuderna-Danish (KD) concentration, nitrogen blowdown, solvent exchange, dilution, and analysis by HPLC-UV. The following recoveries were obtained: ether, 29.2%; benzene, 2.3%; tetrahydrofuran (THF), 6.7%. Based on this study, ether was selected as the solvent providing the best recovery with ease of removal (extract concentration).

The effects of adding concentrated NaOH (1-3 drops) with single or triple sonication were tested with the findings that each of the recoveries was under 3%. These results, performed without soil matrix, indicated that addition of base was detrimental in the absence of the solid matrix.

The source for analyte loss was investigated by checking recovery for each step of the method: 1)ACN with blowdown; 2) ether with blowdown, solvent exchange; 3) ether, KD, blowdown, solvent exchange; 4) 2 g soil sample, 2 g sodium sulfate, 1 sonication, 10 mL ether, KD, blowdown, solvent exchange; 5) 2 g background soil sample, 2 g sodium sulfate, 3 sonications, 10 mL ether, kd, blowdown, solvent exchange; 6) high conc. Method; dilute; analyze. The results were: 1) 75% recovery; 2) 83.5%; 3) 74%; 4) 9.2%; 5) 8.9%; 6) 12.6%. This study indicated that the major source for analyte loss was the sample matrix. Recoveries did not appear to be negatively affected by solvent evaporation to dryness, although that step should be performed carefully, or the extract could be evaporated to near dryness only.

To determine if the soil has a limited capacity for binding the analytes, sand and background soil recoveries were compared, with different spiking concentrations. Sand provided 68%

recovery. Background soil gave under 8% recoveries up to 40 ppm, and 41% at 60 ppm analyte concentration in soil. This study indicated that the soil had a much higher capacity for the target ß-naphthylamine than the action level concentration.

Ammonium hydroxide was added to adjust pH of soil to 11, in an effort to adjust the effect of water in the sample and possibly bind some active sites. Recoveries remained poor at 80-200 ppm analyte concentrations (18-24%).

Aniline was tested as an additive close in structure to the target analytes, yet chromatographically resolvable. The procedures were: (a) 2 g background soil sample spiked with analytes, then with 5 µL aniline; 2 g sodium sulfate, 1 sonication, 10 mL ether as solvent; KD, blowdown, solvent exchange, dilute, analyze. (b) saturate soil with aniline, wash soil with ether to remove excess aniline, weigh out 2 g soil sample, spike with analytes, add 2 g sodium sulfate, 10 mL ether, sonicate once, KD, blowdown, solvent exchange, dilute, analyze. These tests resulted in 20% recovery for (a) and 51% for (b). Thus, it appeared that aniline assisted recovery of target analytes by partial exchange of positions with the analytes on active matrix sites. However, not all sites were apparently subject to exchange, as recovery was significantly better (51% vs. 20%) if all sites were exposed to aniline first.

Comparison to Soxhlet extraction was made using sand and soil matrices with aniline treatment after spiking with the analytes. For this extraction technique, aniline was not found to assist recoverability, and Soxhlet extraction was not an improvement over sonication.

Since aniline was found to improve recoverability of the analytes using ether and sonication, a retest with methanol as solvent was made. In early experiments, methanol had shown some promise. 2 g background soil sample was spiked with analytes and then with aniline, 2 g sodium sulfate, 1 sonication, 10 mL ether or methanol solvent. The recoveries were: ether with 1 µL aniline, 3.9%; ether with 5 µL aniline, 8.6%; ether with 10 µL aniline, 9.5%; methanol without aniline, 2.3%; methanol with 5 µL aniline, 8.9%. Because methanol still did not provide better recoveries than ether, ether was retained as the solvent of choice as the recoveries from ether are less affected by percent water and is easier to remove in the extract concentration step.

Because of the low and poorly reproducible recoveries, silanization of the glassware was performed with dichlorodimethylsilane. Recoveries were much higher: sand, 105%; dry soil without silanization 15% and with silanization, 39%; wet soil with silanization, 27%.

Different amounts of spiked aniline were tested on a 10 g dry background soil sample spiked with 80 µL ß-naphthylamine and processed with 10 g sodium sulfate, 3 sonications, 50 mL ether solvent, KD, blowdown, solvent exchange, dilution and analysis. For 15 µL aniline, the recovery of ß-naphthylamine was 35%; for 25 µL, 52%; and for 35 µL, 47%. These results indicated that there is a threshold concentration above which it provides no significant recovery enhancement, at least in the time period tested (minutes for pre-analysis spikes vs. years for the native contamination).

The possibility of sample drying by oven heating was investigated, but recoveries were not enhanced over those obtained on wet soil. It is known that ß-naphthylamine is volatile in steam, and apparently may be lost by oven drying. Sample cross-contamination could also present a significant problem in the use of oven-drying, so this procedure was not tested further.

As alternative drying procedures to sodium sulfate and oven drying, magnesium sulfate and potassium hydroxide were tested. Magnesium sulfate is an efficient, generally useful neutral drying agent for organic liquids. Potassium hydroxide is often used as a drying agent for liquid amines. 12 g background soil was spiked with analytes, 25 µL aniline, 5 g magnesium sulfate (or 6 KOH pellets), and 50 mL ether solvent; sonicated for 30 sec, allowed to sit for 20 min, resonicated and allowed to sit; sonicated for 60 sec, and allowed to sit for 1 hr. The treated soil was filtered, sonicated twice, and processed by KD, blowdown, solvent exchange, dilution, and analysis. Recoveries were 29% (magnesium sulfate) and 35% (KOH drying). Substitution of a shakeout extraction for the sonication resulted in 23% (magnesium sulfate drying) and 34% (KOH) recoveries.

RESULTS OF SINGLE-LAB TESTING THE FIRST CANDIDATE METHOD

The above preliminary tests and investigations of specific issues resulted in the development of the first candidate analytical method (see below). Several issues remained, however, requiring a careful evaluation of results from the single-laboratory testing of this method. Specifically, reproducibility of the results obtained in the tests of specific issues was relatively poor: recoveries were variable, from ca. 10% to 50%. It was observed that methanol and acetonitrile with sonication dispersed the soil thoroughly, whereas ether and methylene chloride did not break up the soil clumps. Ether and methylene chloride were easy to evaporate for extract concentration, and were poorly water soluble. Methanol was too water soluble to perform well with wet samples (solvent nature changed too much with very wet samples). Acetonitrile had the major disadvantage of poor volatility for solvent volume reduction. Aniline, added

to enhance recoveries by masking active sites on the matrix, fluoresces strongly and HPLC conditions had to be carefully optimized to ensure that all aniline eluted before ß-naphthylamine.

Single laboratory testing demonstrated that the first candidate analytical method could not provide data meeting Region 3 needs with adequate confidence about positive results near or below the action level of 55 ppb. Recoveries again varied from ca. 10% to 50%. A spike allowed to sit on the matrix overnight was not recovered (0% yield).

DEVELOPMENT OF THE FINAL ANALYTICAL METHOD

The first candidate method was changed by applying information obtained in prior testing to arrive at the second candidate method. The second candidate method, in some ways similar to EPA Method 8330 for nitroaromatic explosives, provided more consistent recoveries (ca. 20% to 25% on the background soil). Excellent matrix spike recoveries (1 ppm spikes) were achieved on the Drake Site samples (70-99% on ß-naphthylamine and 72-96% on 4-nitroaniline). These results indicated that the second candidate method provided data of high quality, particularly on the Site samples. This second candidate method, therefore, is the Final Analytical Method.

The second method was designed to address several relevant issues: the low volatility of acetonitrile; the fluorescence of aniline; the activity of the soil matrix; the potential activity of glassware surfaces; breakdown of matrix clumps; improved partitioning of analytes into the organic phase at high pH of the aqueous phase; the need for volatile bases and buffers for thermospray LC/MS; solvent requirements for HPLC.

The second method used the volatile base, concentrated aqueous ammonium hydroxide, to replace KOH and aniline. The method was designed to use a minimum of glassware, with a quantity of acetonitrile that did not require solvent reduction to achieve the desired detection limit. Exposure of the soil to solvent was maximized by the choice of solvent and sonication procedure. The method was relatively fast and straightforward to perform.

FIRST CANDIDATE ANALYTICAL METHOD

Weigh out 10 grams of soil (to 3 decimal places) into a tared porcelain crucible. Dry in an oven at 50°c overnight. Weigh the dried soil to 3 decimal places. Percent water is determined by 10 x (initial weight - final weight).

The following procedure should include a muffle-furnace dried sand blank and a muffle-furnace dried sand laboratory control sample.

Weigh out 10 grams of soil (to 3 decimal places) into a tared, silanized Erlenmeyer flask with a 24/40 ground glass joint opening.

To spike background soil, add 40 µL of a 1000 µg/mL solution of ß-naphthylamine in acetonitrile.

Add 12 pellets of KOH.

Add 100 mL of aniline-treated ether (25 µL aniline in 100 mL ether).

Extract by shake-out for one hour.

When shakeout is complete, decant liquid (without losing soil) into a silanized funnel containing a prewetted (with ether) #41 Whatman filter with a few grams of muffle-furnace dried sodium sulfate (for drying).

Collect the filtrate in a silanized 500-mL round bottom flask with 24/40 ground glass joint opening.

Add 50 mL of aniline-treated ether.

Extract by shakeout for 30 min.

When second shakeout is complete, pour off entire contents of the Erlenmeyer flask into the funnel. Add a few mL of ether to the Erlenmeyer flask to facilitate quantitative transfer of the contents into the funnel. Repeat twice.

Wash the funnel contents with a few ml of ether. Repeat twice.

Attach the round-bottom flask to a rotary evaporator and reduce the volume of the ether solution with vacuum to a few mL.

Remove the round-bottom flask and empty the contents into a 50-mL silanized graduated centrifuge tube.

Add a few mL of ether to the round-bottom flask to facilitate quantitative transfer of the contents into the tube. Repeat twice.

Blow down the solution carefully to dryness with nitrogen.

Bring up the volume to one mL with acetonitrile.

Store extract for analysis.

Analyze by HPLC/fluorescence using conditions outlined in the report.

FINAL ANALYTICAL METHOD FOR ß-NAPHTHYLAMINE AND 4-NITROANILINE IN SOILS AND SEDIMENTS AT THE DRAKE CHEMICAL SITE

Place 2 g soil in a 12 mL vial.

For matrix spikes at 1 ppm, add 200 µL each of 4-nitroaniline and ß-naphthylamine solutions (10 µg/mL).

With disposable pipet, add 10 mL of a solution prepared to be 1% (v/v) concentrated aqueous ammonium hydroxide to 99% acetonitrile (9.6 mL if matrix spike sample).

Sonicate 2 hours at ambient temperature. Hand-shake each 30 minutes.

Centrifuge 30 minutes at 2,000 rpm.

To a new, clean 12 mL vial, add 7 mL 0.01M aqueous ammonium acetate with a volumetric pipet. Add 3 mL of the centrifuged extract. Mix. Filter. Attach a 0.45 mm acrodisc to a 5 mL syringe, push 2 mL through for rinse. Push 1 mL into injection vial.

Rinse syringe with acetonitrile. To avoid cross-contamination, disposable syringes, vials, and pipets are recommended.

Analyze by HPLC/fluorescence using conditions outlined below. Injections of 100 µL were used with an injection loop having a capacity of 250 µL. These conditions provided a detection limit of 1 ppb for ß-naphthylamine and 300 ppb for 4-nitroaniline.

Time (min)	% NH_4OAc (0.01M)	% Acetonitrile
0 (Run #1)	70	30
7.0	70	30
16.0	55	45
16.5	55	45
17.0	0	100
20.0	0	100
20.5	70	30
25.5 = 0 (Run #2)	70	30

APPENDIX A

LITERATURE SEARCH

(ECG) variable that combines temperature, moisture stress, and APAR was used to simulate the daily interactive effects of weather on corn growth and yield. The ECG variable, summed from planting to physiological maturity, was associated with 83% of the variance in grain yields when APAR was estimated with LAI. The ECG variable was associated with 85% of the variance in grain yields when APAR was estimated with the spectral variable GI.

ECONOMICS AND ENERGETICS OF WEED CONTROL IN SOYBEAN TILLAGE SYSTEMS Order No. DA8421019

GOETTE, JOHN MARTIN, PH.D. *The University of Florida*, 1984. 92pp. Chairman: Dr. Wayne L. Currey

Economic and energetic efficiencies of various weed control programs were determined and compared in three soybean [*Glycine max* (L.) Merr.] tillage systems. Field trials were conducted over a three year period between 1981 and 1983 at Gainesville, Florida. Weed control programs consisted of various combinations of preemergence and postemergence herbicide applications. Tillage operations were conventional, minimum, and no-tillage. Weed populations consisted primarily of alexandergrass [*Brachiaria plantaginea* (Link) A. Hitchc.], Florida beggarweed [*Desmodium tortuosum* (SW.) DC.], and redroot pigweed (*Amaranthus retroflexus* L.).

Herbicide treatments included oryzalin applied at 0.84 kg/ha, metribuzin at 0.43 kg/ha, acifluorfen at 0.43 kg/ha, sethoxydim at 0.22 and 0.34 kg/ha, 2,4-DB at 0.28 kg/ha, and glyphosate applied at 1.12 kg/ha.

Cost efficiencies were determined by establishing a cost efficiency ratio (CER = dollar return • production cost^{-1}) for each weed control program within a given tillage system. CER values greater than unity indicated a profitable production system.

The energy efficiency was established by comparisons of energy productivity (EP = yield weight • total energy input^{-1}). An average EP value for United States grown soybeans was approximately 0.1893 kg/MJ.

The comprehensive results indicated that preemergence applications of oryzalin and/or metribuzin were essential for maximizing economic and energetic efficiency. Postemergence applications were usually necessary in order to maintain maximum efficiency; however these treatments were relatively inefficient when they were applied alone.

Directed postemergence applications were the most consistent performing treatments among those tested. The directed postemergence treatment of (metribuzin + 1,4-DB), in combination with preemergence application, produced the greatest single EP and CER across all tillage systems with two exceptions where it yielded the second highest value.

The no-tillage system produced an average EP of 0.083 kg/MJ and CER of 0.39 in 1981. In 1983, no-tillage generated EP and CER values of 0.119 kg/MJ and 0.73, respectively. In 1982, however, the conventional tillage system produced an average EP of 0.10 kg/MJ and CER of 0.44 which were significantly greater than the minimum and no-tillage operations. The variability in tillage system performance was primarily due to the fact that in-row subsoiling was not implemented in 1982 as it was in 1981 and 1983.

REACTIONS OF BENZIDINE, α-NAPHTHYLAMINE AND P-TOLUIDINE IN SOILS Order No. DA8423364

GRAVEEL, JOHN GERARD, PH.D. *Purdue University*, 1984. 171pp. Major Professor: Darrell W. Nelson

Decomposition of ^{14}C-labelled benzidine (4,4'-diaminobiphenyl), α-naphthylamine (1-amino-naphthalene), and p-toluidine (4-amino-toluene) was studied by incubating amine-treated soils for approximately one year and monitoring $^{14}CO_2$ evolved. At the end of incubation, 8.4 to 11.9%, 16.6 to 30.7%, and 18.9 to 35.1% of added benzidine, α-naphthylamine, and p-toluidine, respectively were evolved as CO_2. Degradation rate was described by two first order reactions. Half-lives calculated using the slow phase rate constant were 11.5, 5.3, and 5.0 years for benzidine, α-naphthylamine, and p-toluidine, respectively. Comparison of data from sterile and nonsterile soils suggested that microbes were responsible for degrading amines. Optimum conditions for decomposition were: -0.033MPa water potential, 30°C and pH 5.4. Activation energies calculated from the Arrhenius equation were 40.0, 21.5, and 30.2 kL mol^{-1} for benzidine, α-naphthylamine, the p-toluidine, respectively, suggesting that amine decomposition was biologically mediated. Addition of metabolizable organic substrates did not enhance decomposition.

A batch equilibrium technique was used to study sorption of aromatic amines by soils. Effect of pH on sorption was investigated by comparing amine retention at natural pH to soils having pH values adjusted to 7. Amine sorption increased with a decrease in pH. Sorption isotherms for all compound:substrate combinations were described by the Freundlich equation. Nonlinear sorption isotherms were obtained with benzidine and α-naphthylamine. Equilibrium isotherms for p-toluidine were linear. Freundlich constants (K) for benzidine were correlated with organic carbon content of adjusted soils. There were no significant relationships between soil properties and K values for α-naphthylamine. Freundlich constants for p-toluidine were correlated with % clay in soils for unadjusted and adjusted pH systems. Desorption of sorbed amines followed the order: benzidine << p-toluidine < α-naphthylamine.

The binding of aromatic amines was studied by a sequential extraction of amine-treated soils with ethylacetate:methanol, NH_4OAc and NaOH. Results show amines bind in two phases: initially a reversible equilibrium was established between the amine and soil components. Subsequently, amines become strongly associated with the humic fraction of soil presumably through nucleophilic addition to quinones.

CHARACTERIZATION AND SELECTION OF RHIZOBIA FOR USE AS INOCULANTS FOR GROUNDNUTS IN SUDAN Order No. DA8423708

HADAD, MOHAMED AHMED ELHAG, PH.D. *Iowa State University*, 1984. 127pp. Supervisor: T. E. Loynachan

The abundance of groundnut-nodulating rhizobia in 32 locations in Sudan with variable soil properties did not correlate significantly to either the soil chemical properties or the duration since groundnuts were last planted in crop rotations.

Rhizobium isolates were obtained from six of the commonly grown native legumes in Sudan. The isolates varied in physiological characteristics, serological properties, and nodulating ability on a Sudanese groundnut cultivar. The cultivar showed selectivity for rhizobia isolated from groundnut.

Greenhouse efficiency testing revealed that the commercial strain (TAL 309) was more efficient in nitrogen fixation than either of two Sudanese isolates tested (Wad Medani and Kadugli). The host genotype (Virginia or Spanish) little influenced the efficiency of the strains.

The *Rhizobium* strains TAL 309, Wad Medani, and Kadugli varied in colony morphology, which provided a means of strain recognition of nodule isolates. The method was verified by using serological techniques. Results of a competition study among the three strains showed that the order of competitive ability was TAL 309 > Wad Medani > Kadugli irrespective of the cultivar type.

Increasing the pH from 6.5 to 8.0 in a pot experiment did not influence the competitive ability of the two Sudanese strains when tested on the Sudanese groundnut cultivar 'Ashford'. Raising the inoculation level of the less competitive, but efficient, Kadugli strain by 10^4 times that of the Wad Medani strain gave the Kadugli strain a competitive advantage and resulted in 100% of the main-root nodules being formed by the Kadugli strain.

Under Sudanese field conditions, inoculation of two groundnut cultivars, 'Ashford' and 'Barberton', with selected strains by using different methods did not result in comparable yields to those obtained by adding 120 kg N ha^{-1} as ammonium sulfate. Trends did favor the peat carrier over the oil carrier, and placing the inoculant 10 cm below the seeding depth. The serologically distinct inoculant strain, TAL 309 (applied at 5.3×10^7 viable cells per two groundnut seeds), was fairly competitive with the native rhizobial population present at 2.1×10^4 g^{-1} of soil. Approximately 40% of the nodules were occupied by the inoculant strain.

Decomposition of Benzidine, α-Naphthylamine, and *p*-Toluidine in Soils[1]

JOHN G. GRAVEEL, LEE E. SOMMERS, AND DARRELL W. NELSON[2]

ABSTRACT

Decomposition of ^{14}C-labeled benzidine, α-naphthylamine, and *p*-toluidine in soil was studied in laboratory experiments by monitoring CO_2 production during a 308- to 365-d incubation period. The importance of microbial activity in decomposition of all three aromatic amines was shown by decreased $^{14}CO_2$ evolution in ^{14}Co treated soils. After 365 d of incubation, 8.4 to 12% of added benzidine (54.3 μmol kg^{-1}) was evolved as CO_2, while 17 to 31% of added α-naphthylamine (69.8 μmol kg^{-1}) and 19 to 35% of added *p*-toluidine (93.3 μmol kg^{-1}) were evolved as CO_2 in 308 d. Decomposition of benzidine, α-naphthylamine, and *p*-toluidine was greatest at a water potential of −0.033 MPa and decreased at both 0 and −0.5 MPa. Decomposition was enhanced by increasing the temperature from 12 to 30°C. The absolute amount of α-naphthylamine and *p*-toluidine decomposed increased while the percentage of added amine degraded decreased as application rate was increased from 1 to 1000 mg kg^{-1}. For benzidine, both the amount and proportion decomposed increased with an increase in application rate. Decomposition of aromatic amines was not enhanced by the addition of decomposable substrates. Differences in decomposition of aromatic amines occurred among soils, but consistent relationships between decomposition of amines and soil properties were not observed. In batch equilibration studies, the Freundlich equation described aromatic amine sorption. Isotherms were nonlinear for benzidine and α-naphthylamine and linear for *p*-toluidine. Desorption of sorbed amines followed the order: benzidine < *p*-toluidine < α-naphthylamine and was inversely related to the extent of decomposition.

Additional Index Words: aromatic amines, 4,4′-diaminobiphenyl, 1-aminonaphthalene, 4-methylaniline, sorption.

Graveel, J. G., L. E. Sommers, and D. W. Nelson. 1986. Decomposition of benzidine, α-naphtylamine, and *p*-toluidine in soils. J. Environ. Qual. 15:53-59.

Increased concern over the fate of synthetic organics in the environment has developed within the past few years. Benzidine (4,4′-diaminobiphenyl), α-naphthylamine (1-aminonaphthalene), and *p*-toluidine (4-methylaniline) are synthetic organic compounds which are used in the manufacture of dyestuffs, pigments, rubber products, and agriculture chemicals (Haley, 1975; Golab et al., 1979; USEPA, 1979). These varied applications suggest many possible modes of entry into the environment, ranging from direct discharge to conversion of azo dyes to the parent aromatic amine through bacterial action (Yoshida and Miyakawa, 1973).

Relatively little is known about the fate of benzidine, α-naphthylamine, and *p*-toluidine in soils. Studies by Hauser and Leggett (1940) and Tennakoon et al. (1974) showed that aromatic amines are oxidized rapidly in clay soils to form colored complexes. Sorption of aromatic amines by clay minerals may result in a decrease in biological decomposition. Hsu and Bartha (1973, 1974), Parris (1980), and Bollag et al. (1983) have shown that soil organic matter affects the degradation rates of aromatic amines by covalent bonding of the amino group into humic-type compounds. In contrast, a soil incubation study has shown that benzidine degradation in a silty clay loam soil was rapid with only 20.7% of the parent compound remaining after 4 weeks (Lu et al., 1977). Studies by Medvedev and Davydov (1972) showed *p*-toluidine was decomposed at a faster rate than naphthylamine in a chernozem soil receiving coke industry waste. Hydrolysis of benzidine has been observed in cultures of *Nocardia asteroides* (McClung, 1974). Since additional information is needed concerning the degradation of aromatic amines in soil, the objectives of this work were to determine the effect of soil and environmental conditions on decomposition rates of benzidine, α-naphthylamine, and *p*-toluidine in soil and to assess sorption of these compounds by soils.

MATERIALS AND METHODS

Soils

The soils used in this study were surface (0–15 cm) samples selected to represent a wide range of chemical and physical properties. The field moist soils were air-dried and ground to < 2 mm. Selected properties of the soils are presented in Table 1.

Degradation of Aromatic Amines in Soil

Benzidine, α-naphthylamine, and *p*-toluidine degradation in soil was studied under laboratory conditions, using ^{14}-C compounds: benzidine and *p*-toluidine were uniformly labeled in the ring and α-naphthylamine was labeled at carbon-1. Labeled benzidine (6.0 TBq kg^{-1}) and α-naphthylamine (4.0 TBq kg^{-1}) were obtained from New England Nuclear Company (Boston, MA), and *p*-toluidine (4.5 TBq kg^{-1}) was obtained from California Bionuclear Corporation (Sun Valley, CA). Unlabeled benzidine, α-naphthylamine, and *p*-toluidine were Sigma Technical Grade (St. Louis, MO). Aqueous solutions containing either 5.43 μmol of benzidine (20.1 Bq ^{14}C $mmol^{-1}$), 6.98 μmol of α-naphthylamine (5.9 Bq ^{14}C $mmol^{-1}$), or 9.33 μmol of *p*-toluidine (7.9 Bq ^{14}C $mmol^{-1}$) were added to a 500-mL Erlenmeyer flask and 100 g of soil were added (final soil concentration of amines was 10 mg kg^{-1}). Soils were adjusted to −0.033 MPa water potential by adding distilled water. The contents of each flask were mixed, connected to a closed aeration apparatus and incubated in the dark at 23°C for approximately 1 yr. Humidified CO_2-free air was passed over the soil surface at 0.48 L h^{-1} and CO_2 evolved was absorbed in 25 mL of 1 *M* KOH. The KOH trapping solutions were replaced periodically and analyzed. Periodic measurements of soil moisture indicated that water potential did not vary by more than ± 10% during the experiments.

An aliquot (10 mL) of the CO_2-trapping solution was treated with 5 mL of 1.5 *M* $BaCl_2$ and titrated with 0.5 *M* HCl using phenolphthalein as the indicator to determine total CO_2 evolved. A separate 5 mL aliquot of the KOH trapping solution was acidified with approximately 5 mL of 4 *M* HCl and the released $^{14}CO_2$ was collected in 2 mL of NCS reagent (quarternary ammonium base in toluene; Amersham/Searle Corporation, Arlington Heights, IL) contained in a scintillation vial fitted with an absorption tower. Contents of the tower were washed into the vial with PPO-toluene cocktail (0.5% w/w 2,5-diphenyloxazole in toluene) and the ^{14}C activity determined with a Beckman 7500 scintillation counter. The counts were corrected for background and counter efficiency by assaying a ^{14}C-toluene standard

[1] Contribution of the Purdue Univ. Agric. Exp. Stn., Journal Paper no. 10 000, Dep. of Agronomy, W. Lafayette, IN 47907. Received 25 July 1984.

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Table 1. Properties of soils used in this investigation.

Soil			Particle size distribution			Total			
Series†	Subgroup	pH	Sand	Silt	Clay	C‡	Total N	CEC¶	Water content at −0.033 MPa
			g kg⁻¹				mg kg⁻¹	cmol(+) kg⁻¹	g H_2O kg soil⁻¹
Morocco s	Aquic Udipsamments	4.7	766	213	21	19.1	1667	16.9	123
Oakville s	Typic Udipsamments	5.2	832	127	41	10.4	988	10.2	114
Russell sil	Typic Hapludalfs	5.4	184	692	124	9.8	1111	14.7	244
Chalmers sil	Typic Argiaquolls	5.4	175	643	182	20.8	1975	25.0	185
Milford sicl	Typic Haplaquolls	6.4	43	587	370	27.1	2693	45.4	372
Fox l	Typic Hapludalfs	7.0	483	416	101	19.0	1606	12.8	189
Nappanee sicl	Aeric Ochraqualfs	7.8	161	487	352	18.3	1778	33.4	318

† s, sand; sil, silt loam; sicl, silty clay loam; l, loam.
‡ No inorganic C was present in samples.
¶ Cation exchange capacity obtained from sum of exchangeable Ca, Mg, Na, K, and H.

Table 2. Effect of ^{60}Co sterilization on $^{14}CO_2$ evolution from ^{14}C labeled aromatic amine treated soil.

Aromatic amine	Sterilization†	Incubation time (d)					
		7	14	21	28	35	45
		%‡					
Benzidine	^{60}Co	0.2	0.3	0.4	0.5	0.6	0.7
	Untreated	0.6	1.2	1.7	2.2	2.6	3.3
α-Naphthylamine	^{60}Co	0.0	0.1	0.1	0.2	0.3	0.4
	Untreated	6.2	9.0	11.0	12.6	14.0	15.9
p-Toluidine	^{60}Co	0.1	0.2	0.4	0.7	1.0	1.6
	Untreated	4.5	6.3	7.8	9.1	10.3	11.8

† Samples received either 54.3 μmol benzidine, 69.8 μmol 1-naphthylamine, or 93.3 μmol p-toluidine kg⁻¹ of soil.
‡ Cumulative percent of added ^{14}C activity evolved as $^{14}CO_2$-C.

in a NCS-PPO matrix. A preliminary experiment indicated that benzidine, α-naphthylamine, and *p*-toluidine were not volatilized from aqueous solutions buffered at pH 4, 6, and 8. For all three compounds, < 1% of the amine added was lost through volatilization. This fact together with appreciable sorption by soils indicates that ^{14}C contained in the NaOH trap was $^{14}CO_2$ rather than the amine added.

At the end of the incubation period, soils were air-dried, ground to < 0.15 mm, and assayed for residual ^{14}C activity by the dry combustion method of Cheng and Farrow (1976). Evolved $^{14}CO_2$ was trapped in 50 mL of 1 *M* KOH and ^{14}C was determined as described above. The recovery of benzidine, α-naphthylamine, and *p*-toluidine was based on specific activities of 20.1 Bq mmol⁻¹, 5.9 Bq mmol⁻¹, and 7.9 Bq mmol⁻¹, respectively.

Effect of Incubation Conditions on Decomposition

Soil sterilization was conducted by treating 40 g of Russell soil for 24 h with 2.45 kGy h⁻¹ γ-irradiation from a ^{60}Co source. Gamma irradiation was chosen over autoclaving or chemical sterilization methods because ^{60}Co has a minimum effect on soil properties (Ramsay and Bawden, 1983). After sterilization, the soil was aseptically transferred to sterile 500 mL bottles and treated with an aqueous solution containing either 54.3 μmol kg⁻¹ of benzidine (20.1 Bq ^{14}C mmol⁻¹), 69.8 μmol kg⁻¹ of α-naphthylamine (5.9 Bq ^{14}C mmol⁻¹), or 93.3 μmol kg⁻¹ of *p*-toluidine (7.9 Bq ^{14}C mmol⁻¹). The soil was brought to −0.033 MPa water potential by the addition of sterile distilled water and the bottles were sealed with rubber stoppers fitted with glass rods to which 8 mL vials containing 5 mL of 2 *M* KOH were attached (Bundy and Bremner, 1972). The samples were incubated for 47 d in the dark at 23°C. At regular intervals, the CO_2 trapping solutions were removed, brought to volume in a 10 mL volumetric flask, and the evolved ^{14}C in the KOH was determined by liquid scintillation counting as described above. Periodically the bottles were weighed and sterile, distilled water added aseptically to maintain a constant water potential. Unsterilized samples were treated with ^{14}C-labeled aromatic amines and incubated in the manner described above.

The effects of specific environmental factors were determined by varying one factor while holding all others constant. To evaluate the effect of soil water potential on decomposition, water was added to the Russell soil to maintain water potentials of 0, −0.033, −0.1, or −0.5 MPa. The effect of temperature was evaluated by incubating Russell soil treated with benzidine, α-naphthylamine, and *p*-toluidine at 12, 23, and 30°C. To determine the effect of application rate on $^{14}CO_2$ evolution, benzidine, α-naphthylamine, and *p*-toluidine were applied to the Russell soil at rates of 1, 10, 100, and 1000 mg kg⁻¹ of soil. Glucose and alfalfa (*Medicago sativa*) were added to soils at or before aromatic amine application to determine if degradation of the amines could be enhanced by decomposition of readily metabolizable substrates. Samples of Russell soil were treated with either a single addition of 0.6 mmol glucose at the time aromatic amines were added or multiple 60 mg additions of ground alfalfa forage were made every 7 d for 21 d prior to amine addition. To enrich a population active in degrading amines, aniline (64 μmol) or biphenyl (39 μmol) were added every 7 d for 21 d to incubation flasks containing 60 g of Russell soil prior to treatment with benzidine (54.3 μmol), α-naphthylamine (69.8 μmol), and *p*-toluidine (93.3 μmol) and incubation of 132 d.

Sorption Isotherms

Sorption isotherms were determined in duplicate by equilibrating soil (4 g) with 40 mL of 0.01 *M* $Ca(NO_3)_2$ containing an appropriate concentration of the ^{14}C amine in 50 mL glass centrifuge tubes at 25°C for 24 h in a temperature-controlled shaking water bath. Initial concentrations of benzidine and α-naphthylamine in solutions added to soils ranged from 5 mg L⁻¹ to 200 mg L⁻¹ and *p*-toluidine concentrations were 5 mg L⁻¹ to 100 mg L⁻¹. After equilibration, the liquid and solid phases were separated by centrifugation. Initial and final concentration of benzidine, α-naphthylamine, or *p*-toluidine in the solution phase was determined by liquid scintillation counting on a Beckman 7500 Liquid Scintillation Counter using ACS (Amersham/Searle Corporation) as the counting cocktail. Activity measurements were corrected for counter efficiency by assaying ^{14}C-toluene standards in an ACS matrix. The amount of amine absorbed was calculated by difference between initial and equilibrium concentrations. The pH of the supernatant was determined by glass electrode. Amine decomposition during equilibration was not a problem since < 0.5% of added benzidine, α-naphthylamine, and *p*-toluidine were decomposed in soil in 48 h.

Desorption of benzidine, α-naphthylamine, and *p*-toluidine from soils equilibrated with amines was determined by adding 40 mL of 0.01 *M* $Ca(NO_3)_2$ and shaking the samples for 24 h in a temperature-controlled water bath. The solution phase was separated by centrifugation and the concentration of amine in solution was determined by liquid scintillation counting as described above. The amount of benzidine, α-naphthylamine, and *p*-toluidine occluded in the sediment pellet was calculated from the weight of solution in the pellet and the amine concentration.

Statistical analysis of the data (analysis of variance and Newman-Keul tests, $p = 0.05$) were performed as described by Steel and Torrie (1960). All values are averages of two replications and expressed on an oven-dry soil basis.

RESULTS AND DISCUSSION

Decomposition Under Sterile and Nonsterile Conditions

To determine if benzidine, α-naphthylamine, and *p*-toluidine might degrade by chemical pathways, aromatic amines were incubated in Russell soil which

had been treated with 49 kGy of γ radiation. For all three amines, the $^{14}CO_2$ production rate was significantly higher with the unsterilized soil as compared with the ^{60}Co treated soil (Table 2). Although this behavior supports the premise of microbial metabolism, it is not positive proof of microbial decomposition since small amounts of benzidine, α-naphthylamine, and *p*-toluidine were graded in the "sterile" soil. To assess the sterility of soils incubated for 45 d, soil subsamples were transferred to nutrient broth and incubated. Microbial growth was observed in the broth and thus the soils apparently became contaminated with air-borne contaminants during the incubation period. It is also possible that the limited decomposition was due to chemical hydrolysis. The sterile soil treated with benzidine had approximately 21% of the decomposition observed with nonsterile soil, suggesting that chemical hydrolysis may be partially involved in benzidine degradation in soil. Microbial metabolism is the primary mechanism for degradation of α-naphthylamine and *p*-toluidine.

Degradation of Aromatic Amines in Soil

Evolution of $^{14}CO_2$ was monitored to determine rates of benzidine, α-naphthylamine, and *p*-toluidine decomposition in soils. Since ring-labeled compounds were used, $^{14}CO_2$ release will occur only after ring cleavage, indicating complete degradation of the added amine. It must be realized that measuring $^{14}CO_2$ may underestimate the extent of decomposition if; (i) the compound is only partially metabolized, (ii) the C is incorporated into microbial biomass, or (iii) the amine reacts with soil components (i.e., organic matter) to form stable reactions products. Thus, the decomposition estimates described in this work may be conservative in nature. Decomposition of benzidine was significantly greater in the Chalmers soil (11.6%) than the Russell (10.1%), Nappanee (9.8%), or Oakville (8.3%) soils ($P = 0.05$). The α-naphthylamine degradation rate varied among the six soils studied (Table 3). After 308 d, degradation was greatest in the Russell (30.7%) and Chalmers (28.7%) soils, and was lowest in the Milford soil (16.6%). Decomposition of *p*-toluidine was also influenced by the soil used (Table 3). Degradation was significantly greater in the Fox soil (35%) than the Chalmers (29%), Russell (27%), Morocco (21%), Milford (21%), and Oakville (19%) soils. Other than a correlation between $^{14}CO_2$ release and CO_2 evolution for the *p*-toluidine amended soils ($P = 0.05$), there were no consistent relationships between soil properties and amine degradation rates but it is apparent that soil characteristics are modifying the rate of decomposition.

Total amounts of CO_2-C evolved from the soils treated with benzidine, α-naphthylamine, and *p*-toluidine are presented in Table 3. There was no significant difference in cumulative CO_2 evolved from the unamended Russell (control) and Russell soil treated with the three aromatic amines, indicating that addition of aromatic amines at 100 mg kg^{-1} did not inhibit microbial activity. Since total CO_2 release originates from decomposition of both added amine and soil organic matter and since amines were added at a low rate (equivalent to −7.5 mg C kg^{-1}), it is readily apparent

Table 3. Recovery of ^{14}C activity from benzidine, α-naphthylamine and *p*-toluidine amended soils incubated at 23°C.

Aromatic amine†	Soil	Total CO_2-C evolved‡	Recovery of ^{14}C		
			CO_2-C§	Residual in soil	Total
		mg CO_2 C kg^{-1}	%		
Benzidine	Chalmers	698	11.6a	78.9	90.5
	Nappanee	2146	9.8c	88.0	97.8
	Oakville	736	8.3d	73.1	81.4
	Russell	742¶	10.1b	88.2	98.3
α-Naphthylamine	Chalmers	1094	28.7b	72.2	100.9
	Fox	1301	19.6e	85.9	105.5
	Milford	1006	16.6f	79.2	95.8
	Morocco	596	23.6c	70.9	94.5
	Oakville	714	21.1de	72.7	93.8
	Russell	836#	30.7a	61.7	92.4
p-Toluidine	Chalmers	927	28.7b	74.4	103.1
	Fox	1191	35.0a	59.1	94.1
	Milford	948	20.7ef	78.4	99.1
	Morocco	423	21.2de	62.4	83.6
	Oakville	467	18.9f	72.8	91.7
	Russell	951	27.5c	65.8	93.3

† Incubation period of 365 d for benzidine and 308 d for α-naphthylamine and *p*-toluidine.
‡ Total amount of labeled and unlabeled C evolved from soils.
§ For a specific amine, values followed by the same letter are not significantly different by Duncan's Multiple Range Test ($p = 0.05$).
¶ CO_2-C evolved from untreated soil was 670 mg C kg^{-1}.
# CO_2-C evolved from untreated soil was 963 mg C kg^{-1}.

that total CO_2 evolved is primarily due to decomposition of soil organic matter. As expected, CO_2 release was greater in the high organic matter soils and lowest in the Oakville sand for all three aromatic amines. At the end of the incubation approximately 7.4, 5.4, and 5.1% of the soil organic matter had decomposed from benzidine, α-naphthylamine, and *p*-toluidine amended soils, respectively. Recovery of ^{14}C from soils after incubation for approximately 1 yr indicates that > 90% of the added C can be accounted for as CO_2 or residual ^{14}C in the soil (Table 3). For the majority of experimental systems, the < 30% evolution of $^{14}CO_2$ reflects the recalcitrant nature of the amines, rather than the loss of substrate by volatilization or experimental errors.

The low decomposition rate observed for all three aromatic amines suggests that degradation may be controlled by either soil environmental conditions or by sorption onto soil constituents because the compounds are known to be degraded during sewage treatment.

Effect of Soil Water Potential

Degradation of aromatic amines in Russell soil was greatest at −0.033 MPa for all three amines and it decreased significantly at saturation (0 MPa), −0.1, and −0.5 MPa (Fig. 1). For the benzidine treated soil, very little reduced ^{14}C (e.g., $^{14}CH_4$) was evolved since nearly all of the added ^{14}C-activity was accounted for as CO_2 and residual forms in the soil at the end of the study. The inhibition of benzidine, α-naphthylamine, and *p*-toluidine decomposition associated with a decrease in soil water potential suggests that water availability was a limiting factor. Studies by Parker and Doxtader (1983) showed that 2,4-D decomposition decreased with a decrease in soil water potential. At 0 MPa potential the

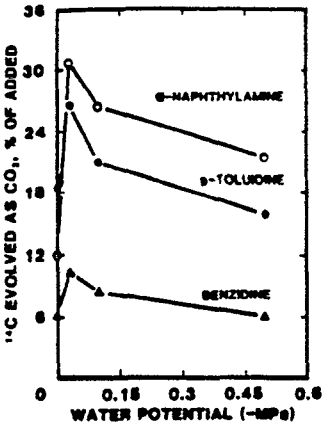

Fig. 1. Effect of soil moisture potential on cumulative $^{14}CO_2$ evolution from ^{14}C-labeled benzidine, α-naphthylamine, and *p*-toluidine amended Russell soil incubated at 23°C.

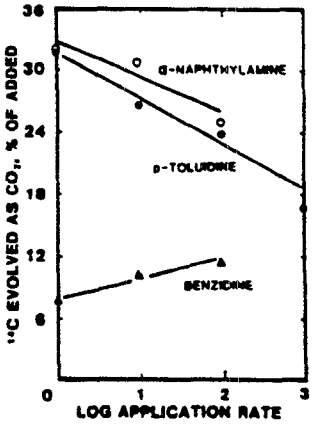

Fig. 2. Effect of amine application rate on cumulative $^{14}CO_2$ evolution from ^{14}C-labeled benzidine, α-naphthylamine, and *p*-toluidine amended Russell soil incubated at 23°C.

soil likely became anaerobic and thus O_2 may limit microbes using a dioxygenase for the initial steps in degradation of aromatic rings (see review by Gibson and Subramanian, 1984).

Effect of Incubation Temperature

As expected for a microbial process, the rates of benzidine, α-naphthylamine, and *p*-toluidine decomposition in the Russell soil were greatly affected by incubation temperature. Decomposition was greatest at 30°C and decreased significantly at 23 and 12°C for all three amines. The decomposition of added benzidine increased from 3.7% at 12°C to 12.2% at 30°C. Approximately 16, 28, and 33% of added α-naphthylamine or *p*-toluidine were decomposed at temperatures of 12, 23, and 30°C, respectively.

The relationship between amine degraded and temperature was described with the Arrhenius equation resulting in activation energies of 52.6, 27.7, and 34.3 kJ mol^{-1} for benzidine, α-naphthyl-amine, and *p*-toluidine, respectively (Table 4). Miekle et al. (1973) suggested that activation energies of 75.4 to 104.8 kJ mol^{-1} indicate chemical degradation while values of ~21 kJ mol^{-1} indicate microbiological reactions. Enzymes have activation energies ranging from 12 to 46 kJ mol^{-1} (Sizer, 1943).

Associated Q_{10} values for benzidine, α-naphthylamine, and *p*-toluidine over the temperature range of 12 to 30°C range from 1.4 to 1.8 (Table 4). The range of Q_{10} values for most enzymatic reactions fall between 1.4 and 2.0 (Meyer and Anderson, 1963). The Q_{10} values found (i.e., ≤ 1.8) support the premise that decomposition of benzidine, α-naphthylamine, and *p*-toluidine was microbial.

Effect of Application Rate

The effect of application rate on the decomposition of benzidine, α-naphthylamine, and *p*-toluidine in Russell soil is presented in Fig. 2. There was a linear relationship between amount decomposed and log of the application rate for all three aromatic amines. The amount of benzidine, α-naphthylamine, and *p*-toluidine decomposed increased with increasing application rate. The percentage of added benzidine decomposed increased from 7.7% at the low rate to 11.4% at the high rate while the reverse trend was found for the other two compounds. The decrease in percent decomposition observed with α-naphthylamine and *p*-toluidine at the high application rates suggests that higher concentrations of the amine may be inhibitory to metabolism or that the rate of the enzymatic reaction has approached a maximum at the high substrate concentrations. The rate of

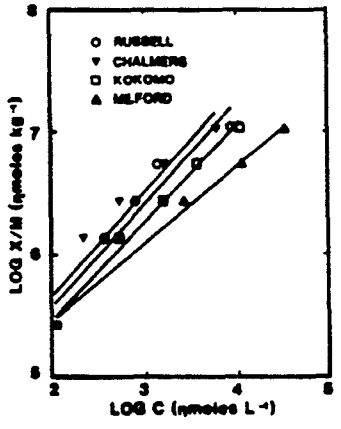

Fig. 3. Sorption isotherms for benzidine retention by soils.

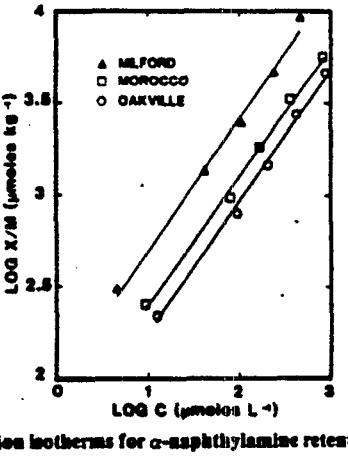

Fig. 4. Sorption isotherms for α-naphthylamine retention by soils.

Table 4. Activation energies and Q_{10} values estimated from temperature variation of benzidine, α-naphthylamine, and *p*-toluidine degradation in Russell soil.

Aromatic amine	Concentration	E	Q_{10}	Correlation coefficient§
	μmol kg^{-1}	kJ mol^{-1}		r
Benzidine†	54.3	52.6	1.8	0.975
α-Naphthylamine‡	69.8	27.7	1.4	0.936
p-Toluidine‡	93.3	34.3	1.5	0.969

† 365-d incubation.
‡ 308-d incubation.
§ Correlation coefficient for log (% decomposed) versus reciprocal of temperature (285, 296, and 303 K).

benzidine decomposition in soils may be substrate limited due to sorption of benzidine by soil constituents. If the solution concentration is below the K_m for enzymes involved in uptake or metabolism, the percent decomposition will increase with an increase in application rate.

Addition of Metabolizable Organic Substrates

Attempts were made to increase the decomposition of benzidine, α-naphthylamine, and *p*-toluidine by adding readily utilizable carbon and energy sources to soil. A single addition of glucose and multiple additions of alfalfa forage had essentially no effect on decomposition of the three amines studied (Table 5). Glucose and alfalfa addition did increase the total CO_2 production from the Russell soil (Table 5).

Decomposition of benzidine, α-naphthylamine, and *p*-toluidine in the Russell soil following preincubation with aniline and biphenyl is also shown in Table 5. There was no significant difference between preincubated and unamended Russell soil for the decomposition of added amine ($p = 0.05$). These findings suggest decomposition of benzidine, α-naphthylamine, and *p*-toluidine will not be enhanced by growth of soil microbes on a readily utilizable carbon and energy source. Enriching for a population capable of decomposing aromatic compounds by adding aniline or biphenyl to the soil also does not promote decomposition of benzidine, α-naphthylamine, and *p*-toluidine. Representative data are shown in Table 5 indicating the nearly complete recovery of added ^{14}C obtained in a range of experimental systems.

Sorption of Amines

Sorption of benzidine, α-naphthylamine, and *p*-toluidine by soils yielded isotherms that could be described by the Freundlich equation

$$x/m = KM^{1/n}$$

where K and $1/n$ are constants, x/m is the amount sorbed per unit weight of soil, and C is concentration in equilibrium solution. By definition, Freundlich sorption isotherms with $1/n$ values = 1 are linear. The logarithmic form of the Freundlich equation was evaluated by linear regression to calculate K and $1/n$. Freundlich sorption isotherms for the amines studied are presented in Fig. 3 to 5. The calculated K, $1/n$, and K_{OC} values for each amine-soil combination are presented in Table 6;

Table 5. Recovery of ^{14}C activity from Russell soil treated with an aromatic amine plus glucose, alfalfa, aniline, or biphenyl and incubated at 23°C for 132 d.

Aromatic amine	Treatment†	Total CO_2-C evolved	Recovery of ^{14}C		
			CO_2-C	Residual in soil	Total
		mg C kg^{-1}		%	
Benzidine	None	56.6	9.4	81.4	90.8
	Alfalfa	77.4	8.2	94.4	102.6
	Aniline	-¶	8.2	86.3	94.5
	Biphenyl	-	8.2	87.5	95.7
	Glucose‡	121	11.0 (10.2)	88.8	99.8
α-Naphthylamine	None	52.1	20.3	79.6	99.9
	Alfalfa	79.3	19.4	83.4	102.8
	Aniline	-	18.5	82.6	101.1
	Glucose§	106	29.7 (30.7)	-	-
p-Toluidine	None	58.7	16.5	75.6	92.1
	Alfalfa	70.8	14.8	77.2	92.0
	Aniline	-	16.3	83.3	99.6
	Glucose§	117	26.6 (26.8)	-	-

† Three additions of 60 mg alfalfa, 1.1 mmol aniline or 0.6 mmol biphenyl to 60 g soil; glucose amendment was a single 0.6 mmol addition to 100 g soil.
‡ 365-d incubation: percent of ^{14}C evolved from soil not treated with glucose shown in parenthesis.
§ 308-d incubation: percent of ^{14}C evolved from soil not treated with glucose shown in parenthesis.
¶ Not determined.

K_{OC} values are calculated as the ratio of Freundlich K/fraction of organic C in soil. If soil organic C is equally effective in sorbing aromatic amines (Lambert, 1968; Brown and Flagg, 1981; Hassett et al., 1981), normalization of sorption constants (K) for organic C content should yield nearly constant K_{OC} values.

The Freundlich sorption isotherms for benzidine were nonlinear as shown by a mean $1/n$ value of 0.768. The values obtained are similar to the mean $1/n$ of 0.515 for benzidine sorption by soils and sediments (Zierath et al., 1980). Values of K for benzidine sorption ranged from 7.6×10^3 to 2.1×10^4. Equilibrium K_{OC} values for benzidine ranged from 227×10^3 to 882×10^3 (Table 6). Even though the sorption of benzidine is correlated with organic C, the strength of the sorption as inferred from K_{OC} is greater than can be accounted for by hydrophobic sorption (Means et al., 1982). Based on the pH of the soil solutions (pH 4.8 to 6.0) and the pKa's of benzidine (pKa = 3.57 and 4.66), benzidine was largely

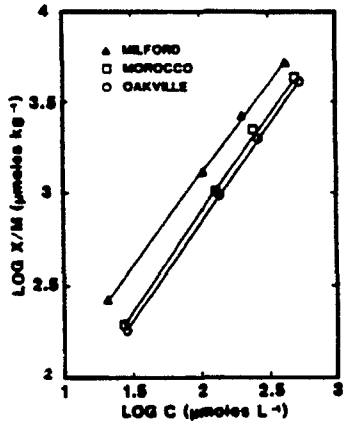

Fig. 5. Sorption isotherms for *p*-toluidine retention by soils.

Table 6. Freundlich sorption constants and K_{OC} values for retention of benzidine, α-naphthylamine, and p-toluidine by soils.

Soil	Equilibrium pH	Freundlich sorption constants† K	1/n	Correlation coefficient (r)	K_{OC}
		Benzidine			
Russell	5.1	8 110	0.834	0.962	882 × 10³
Chalmers	4.8	9 460	0.842	0.950	483 × 10³
Kokomo	5.5	7 600	0.791	0.995	227 × 10³
Milford	6.0	21 000	0.604	0.965	806 × 10³
		α-naphthylamine			
Morocco	3.9	49.7	0.704	0.998	2 688
Oakville	4.2	34.3	0.717	0.996	3 173
Milford	5.9	98.2	0.714	0.996	3 777
		p-toluidine			
Morocco	4.0	5.97	1.062	0.999	323
Oakville	4.3	5.36	1.061	0.999	496
Milford	5.9	13.21	0.992	0.999	508

† Freundlich molar sorption constants and correlation coefficients for benzidine determined from log x/m (nmol kg⁻¹) plotted against log C (nmol L⁻¹), α-naphthylamine and p-toluidine determined from log x/m (μmol kg⁻¹) plotted against log C (μmol L⁻¹).

present as the neutral species in the soils studied. Some sorption of cationic benzidine could have occurred, but it is likely that benzidine present as a neutral species was adsorbed to organic matter through hydrophobic bonding (Zierath et al., 1980).

Small amounts (< 1%) of sorbed benzidine were desorbed with 0.01 *M* $Ca(NO_3)_2$ over the range of concentrations and soils studied (data not presented). Benzidine is tightly bound by soil constituents within a short period of time. The irreversible binding could result from several reactions including exchange of benzidine with an adsorbed inorganic cation on montmorillonite (Lahav and Raziel, 1971), adsorption and oxidation of benzidine following interaction with clay minerals (Tennakoon et al., 1974) and covalent bonding with soil organic C (Parris, 1980; Bollag et al., 1983).

The Freundlich sorption isotherms for α-naphthylamine were nonlinear as indicated by an average $1/n$ value of 0.712 with K values ranging from 34.3 to 98.2. The pH of the soil solution and the pKa for α-naphthylamine (pKa = 3.92) indicate that α-naphthylamine was present as a neutral species in most soil systems, and thus sorption was likely the result of hydrophobic interactions with the organic component of soils.

Desorption of α-naphthylamine from the Morocco, Oakville, and Milford soils ranged from 7 to 21%, 9 to 27%, and 1 to 18%, respectively, and the proportion desorbed always increased as the amount sorbed increased. These data suggest α-naphthylamine is retained through both cation exchange reaction with clays and organic matter in acid soils and through physiochemical reactions with organic matter at near neutral pH. Furthermore, a significant percentage of the sorbed amine can be readily released into the soil solution and subjected to decomposition.

Sorption isotherms for p-toluidine were linear with $1/n$ values approaching unity (Fig. 5) while K values ranged from 4.77 to 13.21. The K values were correlated with clay content (r = 0.997). The calculated K_{OC} values ranged from 323 to 508. Since the pKa for p-toluidine is 5.08, appreciable concentrations of protonated and neutral species will be present in the Morocco, Oakville, and Milford soils, suggesting that a large proportion of p-toluidine was retained by clay minerals and organic matter through cation exchange. Desorption of sorbed p-toluidine was a relatively constant fraction of sorbed p-toluidine in all soils varying from 11.3 to 15.8%. These data suggest that p-toluidine is likely held as an exchangeable ion by the clay and organic fraction of soils.

SUMMARY

The results obtained from this study indicate that benzidine, α-naphthylamine, and p-toluidine are slowly degraded in soils as shown by CO_2 evolution. Chemical hydrolysis may also play a role in benzidine decomposition because sterile soils had 21% of the decomposition observed under nonsterile conditions.

Soil environmental conditions had only a limited effect on the decomposition of aromatic amines. Modifying soil environmental conditions such as water potential, temperature, or addition of metabolizable organic substrates will not markedly affect the short-term rate of amine degradation to CO_2 in soils. Sorption experiments indicated that benzidine was strongly retained by all soils studied and that minimal amounts of sorbed benzidine were desorbed by a neutral salt. In contrast, both α-naphthylamine and p-toluidine were not only less tightly bound but also more desorbable than benzidine. It is suggested that one factor responsible for differing rates of degradation in a range of soils is related to the extent and strength of sorption.

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Nitrogen Transformations in a Poultry Manure Amended Soil: Temperature and Moisture Effects[1]

J. T. SIMS[2]

ABSTRACT

Efficient agronomic management of poultry manure requires information on its contribution to the N budget of the soil-crop system. Of primary importance in this regard is the timing of N mineralization from the organic fraction of the manure. As manure applications are frequently made year round, it is also essential to understand how environmental variations will alter the rate and extent of N mineralization. Consequently, a laboratory study was conducted for 150 d to measure the effects of temperature and moisture on net N mineralization from three poultry manures. Each manure sample was incorporated with an Evesboro loamy sand (mesic, coated typic Quartzipsamments) and changes in soil inorganic N (NH_4^+-N and NO_3^--N) and pH were determined at 30, 90, and 150 d. Although variations were noted between manure types, most of the net mineralization (N_m) observed at 25 °C and 40 °C occurred within the first 90 d. Mineralization was reduced at 0 °C but, by the conclusion of the study, considerable accumulations of inorganic N (18–76 mg/kg) were detected at this temperature. Thirty to 60% of the organic N added to the soil in manures 1 and 3 was mineralized, when moisture was not limiting. Inconsistent mineralization patterns obtained with manure 2 were attributed to microbial immobilization of N. Accumulations of NH_4^+-N under cold or dry conditions resulted in elevated soil pH levels. Conversely, considerable soil acidification occurred under warm, moist conditions due to rapid nitrification of NH_4^+-N added in the manures.

Additional Index Words: **mineralization, nitrification, organic N.**

[1] Published with the approval of the Director of the Delaware Agric. Exp. Stn. as Misc. Paper no. 1109. Contribution no. 188 of the Dep. of Plant Science, Univ. of Delaware, Newark, DE 19717-1303. Received 4 Mar. 1985.

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The Delmarva (Delaware–Maryland–Virginia) peninsula is one of the most concentrated areas of poultry production in the USA. In the state of Delaware alone, approximately 245 × 10^6 kg of poultry manure are generated annually. This manure is used primarily as a fertilizer material for the 50 000 ha of corn and small grains grown in the southern-most county of the state. Much of this grain is produced on deep, easily leached, sandy soils. Consequently, agricultural utilization of poultry manure must be tempered with an awareness of the potential environmental problems associated with nitrate contamination of groundwater. Recent reports of such contamination (12, 20) prompted this investigation into improved nitrogen (N) management of poultry manure.

Efficient management of poultry manure as a N source requires a knowledge of several aspects of its transformations in the soil. It is first necessary to characterize the manure in terms of its total contribution to the soil N budget. More complex, but of greater importance is the need to estimate the percentage of this contribution that will be available for crop use during the current growing season. Perhaps most important is the timing, within the growing season, of N release from the organic fraction of the manure. Growers who supplement poultry manure with fertilizer N must know when the manure N will be available in order to maxi-

Si:P ratios >1000. Thus, special care must be exercised to avoid a silica bias.

More recently, P determinations by using popular "Hach Kits" have yielded soluble phosphate estimates an order of magnitude higher than accurate *total*-P estimates in the same uncontaminated cold spring water (Stauffer, unpublished). In the total-P determination a silica bias failed to develop because of retained acidity from the persulfate digestion step. This carried over into the colorimetric step and raised the H^+:Mo ratio; the lower ratio in the soluble-P determination allowed the development of a silica bias in that silica-rich groundwater.

Elsewhere, P determinations for hot spring drainage waters in Yellowstone National Park published prior to 1979 were biased by up to *3 orders of magnitude* (*11*, *12*). Phosphate-P concentrations in silica-rich lake water and groundwaters of East Africa have been reported (*15*, *16*, *28*, *29*) ranging up to *50 mg* L^{-1} *P*. However, some of these estimates have not been substantiated by radio-tracer methods (*30*). Until further checked for a possible silica and/or As bias, these reported P concentrations in volcanic regions should be treated with caution.

ACKNOWLEDGMENT

Both James Ball and David Johnson made helpful criticisms of the draft manuscript. This paper is dedicated to the statistician G. E. P. Box.

Registry No. Arsenic, 7440-38-2; molybdenum blue, 66771-43-5; silica, 7631-86-9; water, 7732-18-5.

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RECEIVED for review January 20, 1983. Accepted April 15, 1983. Partial financial support was provided both by the U.S. Geological Survey and by the U.S. EPA Research Grant R805281010.

Cation-Exchange Concentration of Basic Organic Compounds from Aqueous Solution

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A macroreticular poly(styrene–divinylbenzene) cation-exchange resin is prepared. An aqueous sample is passed through a column of this resin in the H^+ form and organic bases are taken up as cations. Washings with methanol and ethyl ether remove sorbed neutral and acidic compounds. Ammonia gas is introduced into the column prior to elution of the basic organics with either methanol or ethyl ether containing ammonia. The eluate is concentrated by evaporation, and the concentrated sample is analyzed by gas chromatography. Over 50 organic bases are recovered from water at the 1 ppm and 50 ppb levels. Recoveries of over 85% are achieved for most of the compounds studied with at least one of the eluents. The resin procedure shows improved recoveries and reproducibility over a simple ether extraction procedure. Real samples of river water, shale process water, and supernatant from an agricultural chemical disposal pit are analyzed for basic organic material by using the resin procedure.

The analysis of complex aqueous mixtures of trace organic compounds usually involves preliminary isolation and concentration steps (*1*–*6*). Any preliminary separation should be selective, while minimizing analyte loss. This separation may consist, entirely or in part, of a division of the compounds of interest into one of several general classes (*3*, *4*). This classification is usually based on a particular compound characteristic, such as hydrophobicity or acidity. A preconcentration step is often necessary to permit direct analysis by conventional methods without appreciable loss of sensitivity (*5*, *6*). The transfer of the analytes from aqueous solution to an organic solvent also facilitates the use of several common analytical techniques, such as gas chromatography. An ideal preparative method for organics in aqueous solution would combine these processes (isolation, concentration, transfer) into a single step.

Many techniques have been developed to simultaneously fractionate and concentrate organics from aqueous solution.

Solvent extraction has been used extensively for various types of organics (*7–9*). However, such procedures can be tedious, especially if a large aqueous sample is to be extracted with a small amount of organic solvent. In addition, the distribution coefficient of the analyte is not always favorable. Some compounds, especially those exhibiting a significant degree of hydrophilicity, may not extract well.

Methods such as headspace analysis (*9, 10*), gas stripping (*11–13*), and distillation (*14, 15*), can also be used to isolate and concentrate aqueous organics. These methods are, however, generally restricted to volatile, low molecular weight compounds.

The sorption of organics on a solid matrix is also an effective concentration technique. Activated carbon (*16, 17*), porous polymers (*18*), and other materials (*19–21*) have been used as sorbents. While activated carbon is a common sorbent, it is often difficult to remove the sorbed organics from the carbon matrix. The effectiveness of macroreticular poly(styrene–divinylbenzene) resins for sorbing organic compounds from aqueous solutions has been demonstrated (*3, 22–24*).

Richard and Fritz (*25*) developed an excellent method for the isolation and concentration of organic acids and acid anions from aqueous samples. The organic anions are retained on a small column containing macroporous anion–exchange resin. After sorbed neutral organics are washed off of the resin, the anions are converted to the molecular form and eluted from the column with HCl gas in ethyl ether.

Now, a similar scheme employing a cation-exchange resin is used to isolate basic organic compounds from aqueous samples. The sorption of organic bases by cation-exchange resins is well documented (*26–30*). A cation-exchange method has been used to concentrate pyridines from beers and worts (*31*). In the present work a macroporous cation exchanger in the hydrogen form is used to retain organic bases as cations from aqueous samples. Neutral organic compounds that might be sorbed by the resin are removed by washing with methanol and ethyl ether. The protonated bases are then converted to their free base forms by passing ammonia gas through the column and are eluted with either ammonia-saturated methanol or ammonia-saturated ethyl ether. After careful evaporation, the individual bases are separated by gas chromatography.

EXPERIMENTAL SECTION

Cation-Exchange Resin. A cation-exchange resin was prepared from the macroreticular poly(styrene–divinylbenzene) copolymer XAD-4 (Rohm & Haas, Philadelphia, PA). The resin was ground and dry sieved. The 80–100 mesh fraction was retained and sequentially Soxhlet extracted with methanol, tetrahydrofuran, acetonitrile, acetone, and ethyl ether for 10 h each. The purified resin was then dried overnight at 50 °C.

The XAD-4 resin was sulfonated by adding a 10-g sample of resin to 200 mL of concentrated (96%) sulfuric acid and 100 mg of Ag_2SO_4 that had been heated to 110 °C. The reaction mixture was stirred for 2.5 h and then allowed to cool to room temperature. When cool, the reaction was quenched by pouring the reaction mixture into 2 L of distilled water with ice. After quenching, the resin was filtered off and placed in a 15 cm × 8 mm i.d. Pyrex column with a stopcock at the bottom. The resin was flushed with 2 N H_2SO_4 until the effluent was free of Ag^+ ions. The sulfonated resin was removed from the column and Soxhlet extracted for 10 h each with methanol, acetonitrile, and ethyl ether. The resin was returned to the column and alternately changed from the H^+ to the Na^+ form several times with 2 N NaOH and 2 N HCl. Finally, the resin, in the H^+ form, was washed with 100 mL each of distilled water, methanol, and acetone, and dried overnight at 50 °C.

The capacity of the sulfonated XAD-4, as determined by direct and indirect titration, was 2.86 mequiv/g.

Concentration Apparatus. The columns, reservoirs, and three-plate Snyder distillation columns used are described elsewhere (*3, 22*). The column dimensions were 15 cm × 8 mm i.d., and the reservoirs were of 250 mL and 1 L capacity. Concentration flasks used were those described by Junk et al. (*22*), and were of 100 mL capacity.

The inner surfaces of all reservoirs were deactivated (to minimize adsorption of analyte compounds on the glass) by treatment with a 5% (v/v) solution of dimethyldichlorosilane in toluene. Deactivation of the columns or the concentration flasks was not performed.

Gas Chromatography. The recovery studies were performed with a Tracor 550 gas chromatograph, with both packed and capillary columns, using an FID detector. The packed columns used for most of the recovery studies were 2 m × 4 mm i.d. glass columns packed with 10% Carbowax 20M/2% KOH on 80–100 mesh Chromosorb W AW. Several high-boiling basic compounds and most of the neutral compounds were separated by using the same columns packed with 3% OV-17 on 100–120 mesh Supelcoport. Capillary columns were fused silica, 30 m × 0.25 mm i.d., coated with SE-54. Analysis of the natural samples was performed on a Hewlett-Packard 5794A gas chromatograph using a 27 m × 0.25 mm fused silica capillary column with a thick film (1.0 μm) of the bonded stationary phase, DB-5.

Reagents and Chemicals. All solvents except methanol were reagent grade. Methanol was "distilled-in-glass" grade from Burdick and Jackson (Muskegon, MI). All solvents except ethyl ether were used as received. The ethyl ether used in the elution procedure was specially purified by sequential extraction with one-tenth volume each of 1 M $NaHSO_3$, saturated NaCl solution, NaCl saturated 1 N NaOH, NaCL saturated 1 N H_2SO_4, and finally twice with saturated NaCl solution. The washed ethyl ether was then distilled from CaH_2 and molecular sieves just prior to use. The purity of the solvent was checked periodically by blank analysis.

Anhydrous ammonia gas had a purity rating of 99.99% and was purchased in lecture bottles from Matheson (Joliet, IL). All other chemicals were reagent grade or better. Model compounds and other reagents were used without further purification.

Water used in the recovery studies was purified by passage through a multibed purification system manufactured by Barnstead (Boston, MA).

Cation-Exchange Procedure. Allow a 2.45-g resin sample (total capacity 7 mequiv) to stand in pure water for 2 h and then add resin slurry to a column plugged with silanized glass wool. Add the aqueous sample to the column from a reservoir attached to the top of the column by using a gravity flow rate of approximately 3 mL/min. After sample passage is complete, rinse the reservoir twice with 20 mL of water and allow each washing to enter the column and drain. Blow out the column briefly with a gentle stream of air. Reattach the reservoir, rinse the walls with 10 mL of methanol, and allow the methanol to completely fill the column. Cap the column and shake to remove entrapped air. Allow the methanol level to drain to the top of the resin bed and then elute the column with a second 10-mL portion of methanol. Gently blow out the methanol from the column with a stream of air. Without reattaching the reservoir repeat the washing procedure with a 10-mL portion of ethyl ether, followed by a 15-mL portion.

Place a concentration flask beneath the column and blow out the residual ether with a slow stream of ammonia gas. Continue passing ammonia gas until about the top 90% of the column has become lighter brown in color, indicating conversion of the resin sites from H^+ to NH_4^+. Stop the ammonia gas flow and add 10 mL of eluent saturated with ammonia gas to the column. (This is prepared by bubbling ammonia through cooled solvent for about 10 min.) When the liquid level reaches the top of the resin bed, add a second 10-mL portion of NH_3 eluent, cap the column, and shake gently to remove entrapped air. Pass the remaining solution plus a final 20-mL portion of NH_3 eluent through the column. Concentrate the 40 mL of effluent to approximately 1 mL according to the procedure used by Junk et al. (*22*). Use a portion of the final 1 mL concentrate for gas chromatographic analysis of the individual sample bases.

Regenerate the resin column immediately after elution by sequential washing with 30 mL of NH_3 eluent, 50 mL of pure water, 50 mL of 3 M hydrochloric acid, and 150 mL of 1 M sulfuric acid. Finally, flush the column with pure water until the effluent is neutral.

Procedure for Recovery Studies. To prepare samples of known solute concentration for recovery studies, add the desired amount of water to the reservoir and then add a calculated amount of a standard solution from a syringe to produce a water sample [redacted] the desired final concentration. Swirl to mix the solution. Carry [redacted]piked sample through the cation-exchange concentration, [redacted]poration, and gas chromatographic steps as outlined above. Calculate the percentage recovery by comparing the peak heights (or peak areas) of the spiked water sample with those obtained by adding the same volume of standard solution to 1 mL of the eluting solvent and chromatographing directly. All chromatographic peaks were normalized to an internal standard.

Solvent Extraction Procedure. Extract an aqueous sample, spiked with known concentrations of model organic compounds, with four 10-mL aliquots of purified ethyl ether. Discard the aqueous layer and remove the last traces of water from the combined extracts by freezing in a bath of liquid nitrogen. Transfer the 40-mL extract to a concentration flask, concentrate as in the resin procedure, and calculate the percentage recoveries after separation by gas chromatography.

Real Samples. To prepare river water samples, filter 2-L aliquots through a medium grade glass frit to remove particulates. To avoid possible adsorption on the glass collection vessels, analyze samples as soon as possible after collection. Load columns with 5 g of resin, and allow gravity feed. Wash with 50 mL each (2 × 25 mL) of methanol and ethyl ether to ensure complete removal of neutrals. For the remaining procedure, follow that used for the model compounds recovery study. After GC/MS analysis of the 1 mL concentrated sample, reduce the volume further to 0.1 mL with a stream of dry helium, and rechromatograph to enhance peaks of minor components.

Use the same procedure for disposal pit sample, but filter a 300-mL aliquot though a Celite cake (0.5 cm depth) and fine-grade glass frit. Dilute this with 700 mL of pure water for a 1 L total sample. Reduce this sample also 0.1 mL after GC/MS analysis [redacted]rechromatograph.

[redacted] shale process water sample was run without filtration, since [redacted]rticulates were present. Dilute a 50-mL sample with 50 mL of pure water and run through the same procedure as that for the previous real samples.

RESULTS AND DISCUSSION

Development of Method. The general scheme for the selective concentration and determination of basic organic compounds in aqueous samples is as follows. A measured volume of the aqueous sample is passed through a small column filled with a special cation-exchange resin in the hydrogen form. Basic compounds take up a proton and are retained by the ion exchanger as a protonated amine cation. Many neutral organic compounds are also taken up by the ion-exchange resin by sorptive effects, but these are removed by washing the resin column with methanol and then with ethyl ether. Next, the interstitial solvent is blown out and ammonia gas is passed through the column to neutralize the hydrogen ions of the ion exchanger and partially convert the organic amine cations to the free, molecular bases. The organic bases are then eluted from the column with an organic solvent saturated with ammonia gas. The effluent is carefully evaporated almost to dryness and a portion is injected into a capillary-column gas chromatograph in order to separate the individual sample components.

In previous work in which an anion-exchange resin was used to selectively retain anions of organic acids, quaternized XAD-4 was found to work much better than the other resins tried (25). Gel-type resins and commercial macroporous resins [redacted]und to partially disintegrate after repeated swelling [redacted]inking in the columns used. Because of this experience, the cation-exchange resin used in the present work was prepared by sulfonating XAD-4. Sulfonation is very easy to perform and the resulting ion-exchange resin has performed all in all respects.

In the washing step, methanol removes from the column and elutes (at least in part) neutral organic compounds sorbed by the resin. The subsequent wash with ethyl ether completes the elution of neutral organics and washes out the residual methanol.

At first, only solvent (ethyl ether or methanol) saturated with ammonia gas was used to convert the sample cations to neutral species and elute them from the column. However, it quickly became apparent that this procedure was not sufficiently reproducible. It is difficult to prepare reproducible solutions of ammonia in either solvent. Elution of sample components varies considerably from one run to another unless a large colume of the eluent is used. However, a large volume of eluent necessitates a longer time for evaporation and the blank is also increased. The prior addition of ammonia gas to the column, followed by elution of sample compounds with a rather small volume of ammonia in solvent nicely avoids the difficulties mentioned above. Neutralization of the hydrogen ion function of the exchanger with ammonia gas is followed easily by a change in color of the resin column.

When this research was begun, only packed chromatographic columns or coated glass capillaries were available for the gas chromatographic separation of various sample bases. This severely limited the chromatographic resolution that could be attained. However, the fused silica GC column with a chemically bonded stationary phase provided easy separation and excellent resolution of most base mixtures.

Recovery Studies. Recoveries for a wide variety of model organic compounds by the cation-exchange procedure are presented in Table I. The recovery for each compound was determined at the 1 ppm and 50 ppb concentration levels from 100-mL and 1-L samples, respectively. The following conclusions seem justified:

Virtually all of the basic compounds tested give good to excellent recoveries with at least one of the eluents. Indole and carbazole do contain nitrogen but they are hardly basic at all and therefore give close to zero percentage recoveries. Diphenylamine is also a very weak base and might be expected to give a low recovery.

Several basic compounds give significantly higher recoveries when ammonia in methanol is used as the eluent. This is a consequence of the higher solubility of ammonia in methanol and perhaps of the better solvating properties of methanol, compared with ethyl ether. Distilled-in-glass methanol is available which requires no further purification, while ether must be further purified before use. However, the greater volatility of ethyl ether and its superior properties as a solvent for GC samples make it the eluent of choice for those compounds where the recovery is satisfactory.

The lower recoveries of some aliphatic amines and *o*-phenylenediamine are due at least partially to losses during the concentration and evaporation step. Spiked 40-mL samples of ethyl ether showed significant losses of *n*-hexylamine, *n*-octylamine, and *o*-phenylenediamine when concentrated to 1 mL with a Snyder column. It is possible that losses of some aliphatic amines could occur by irreversible adsorption on active sites of glass columns and concentration vessels. The improved recoveries with ammonia in methanol support this hypothesis because adsorption would be reduced in the more polar solvent.

The selectivity of the cation-exchange concentration for basic compounds was checked by running several neutral organic compounds through the procedure. With either eluent, the recoveries of ethylbenzene, *o*-dichlorobenzene, indene, napthalene, methyl benzoate, and diethyl phthalate were essentially zero at 1 ppm initial concentration. In addition, acetal, hexanal, bromoform, *n*-decane, mesitylene, and 2-ethyl-1-hexanol gave zero percent recoveries at 1 ppm using the NH_3 ether eluent. This selectivty is probably a consequence of the washing step because at least some neutral

Table I.

Table I. Recovery of Basic Compounds from Aqueous Samples by the Cation-Exchange Method[a]

compound	recovery, %			
	NH_3–ether 50 ppb[b]	NH_3–ether 1 ppm[c]	NH_3–CH_3OH 50 ppb[b]	NH_3–CH_3OH 1 ppm[c]
Aliphatic Amines				
hexylamine	34	46	80	85
cyclohexylamine	35	47	79	84
octylamine	38	45	80	91
2-ethylhexylamine	34	48	72	98
diallylamine	84	86	88	86
dipropylamine	33		83	87
dibutylamine	89	93	99	99
dihexylamine	90	86	84	95
N-methylcyclohexylamine	91	91	99	97
dicyclohexylamine	84	88	86	95
triallylamine	88	88	89	91
tributylamine	89	93	91	96
Aromatic Amines				
aniline	88	90	95	95
o-toluidine	88	87	88	96
m-toluidine	87	99	89	99
o-chloroaniline	83	85	82	97
p-chloroaniline	84	92	83	97
2,5-dichloroaniline	65	92	79	92
N-methylaniline	90	90	98	98
N,N-dimethylaniline	86	88	99	99
N,N-diethylaniline	91	89	96	97
diphenylamine	10	15	46	48
1-naphthylamine	80	74	80	81
o-phenylenediamine	35	69	39	71
Heterocyclic Compounds				
pyrrolidine	<3	6	65	71
piperidine	6	17	93	93
morpholine	46	67	99	98
N-methylmorpholine	93	99	92	99
piperazine	<3	4	86	92
imidazole	<3	10	91	100
3-methylpyrazole	44	78	87	97
3,5-dimethylpyrazole	45	78	91	97
indole	0	0	0	0
carbazole	<3	4	<3	4
pyridine	76	87	95	95
2-picoline	87	89	94	98
4-picoline	96	90	95	99
4-isopropylpyridine	88	88	92	96
2,4-lutidine	90	91	91	94
2,6-lutidine	98	98	98	97
2,4,6-trimethylpyridine	90	88	100	100
2-aminopyridine	40	60	89	99
2,2′-bipyridyl	86	100	90	100
quinoline	90	97	99	99
isoquinoline	87	100	99	100
quinaldine	88	87	97	97
2,6-dimethylquinoline	90	90	97	98
acridine	90	90	88	89
benzo[*h*]quinoline	90	91	95	96
benzo[*f*]quinoline	92	93	98	98
2-methylpyrazine	83	88	93	96
quinoxaline	77	76	99	94
2,3-dimethylquinoxaline	84	85	90	95

[a] Column is 90 × 8 mm filled with sulfonated XRD-4; total capacity is 7 mequiv. All recoveries are an average of four runs. [b] 1 L sample. [c] 100 mL sample.

organic compounds are known to be taken up on the ion-exchange resin. Dichlorobenzene was detected in the concentrated methanol wash during the analysis of a mixture of dichlorobenzene and several organic bases.

Solvent Extraction Studies. For several model compounds, a spiked aqueous sample was extracted four times with ethyl ether. The combined extracts were evaporated and the solutes separated by gas chromatography. The recoveries of these model compounds are compared with recoveries obtained by the ion-exchange procedure in Table II. A higher recovery (and better precision) was obtained in every case the ion-exchange method except for indole, which is really basic and should give zero recovery in the ion-exchange procedure. The low recovery of imidazole could be due to its high polarity (in the solvent extraction me[illegible] evaporation.

Analysis of Real Samples. Generally, the amount and variety of basic compounds found in natural water samples

Table II. Comparison of Recoveries with Solvent Extraction and Resin[a]

compound	recovery, % solvent extraction	recovery, % resin procedure
tributylamine	73 ± 4	93 ± 4
2,4,6-trimethylpyridine	72 ± 6	88 ± 1
dihexylamine	64 ± 5	86 ± 1
N,N-diethylaniline	65 ± 8	89 ± 3
N-methylaniline	78 ± 7	90 ± 4
o-toluidine	81 ± 7	87 ± 2
2-aminopyridine	12 ± 2	60 ± 2
quinoline	83 ± 6	97 ± 3
isoquinoline	79 ± 9	100 ± 3
imidazole	2 ± 3	10 ± 4
2,5-dichloroaniline	78 ± 8	92 ± 5
indole	75 ± 12	0 ± 0

[a] All at 1 ppm in 100 mL of solution.

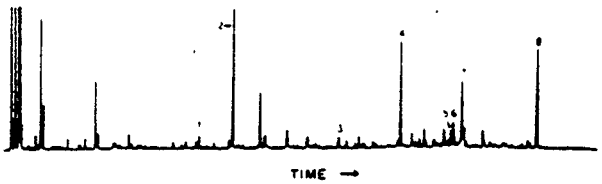

Figure 1. FID chromatogram of compounds isolated from an agricultural chemical disposal pit: sample concentrated 3000-fold; GC conditions, 27 m × 0.25 mm I.d. fused silica capillary, 1 μm DB-5; held at 50 °C for 4 min and then programmed at 5 °C/min to 250 °C and held for 10 min; injection volume was 2 μL split 100:1, with helium carrier gas. Compounds identified were (1) *N,N*-dimethylcyclohexylamine, (2) acetophenone, (3) Dichlobenil, (4) 1-naphthylamine, (5) Simazine, (6) Atrazine, (7) Trietazine, and (8) Diphenamid. An asterisk indicates compounds not confirmed by retention time matching.

are much less than for acids or neutrals (*32*). Basic material may be adsorbed on particulate matter in the water or associated with the humic and fulvic acids usually present. In addition, there are fewer natural sources of basic materials, so the presence of organic bases in a natural water system can usually be attributed to an outside source. As an example, the recent discovery of aromatic amines in the Buffalo River was attributed to the effluent from a nearby dye manufacturing plant (*33*).

Our analyses of water samples from the Skunk River (IA) and the Delaware River (PA) also showed little basic material. The chromatogram of the Delaware River extract showed three small peaks, two of which were tentatively identified as pyrazole and a methylethylpyridine isomer by GC/MS analysis. The third peak was unidentified. Nicotine was the only compound identified by GC/MS in the Skunk River sample. A second 2-L aliquot of the same sample again showed the presence of nicotine, and some benzo[*h*]quinoline. A third aliquot was analyzed to check for these compounds, but only nicotine was found. Several other small peaks seen in the chromatograms remain unidentified. While a quantitative analysis was not performed, a rough estimate of the nicotine concentration would be below 50 ppb.

The gas chromatogram of compounds isolated from a sample of supernatant water from an agricultural chemical disposal pit after concentration by the ion-exchange procedure is shown in Figure 1. Many of the compounds present have been identified, at least tentatively, by GC/MS analysis. The compounds identified are the following: 1-naphthylamine, Simazine, Atrazine, Dichlobenil, Diphenamid, *N,N*-dimethylcyclohexylamine, and Trietazine. All but the last two have been confirmed by matching retention times with those of authentic samples. In addition, acetophenone was identified, which of course contains no nitrogen. The herbicide

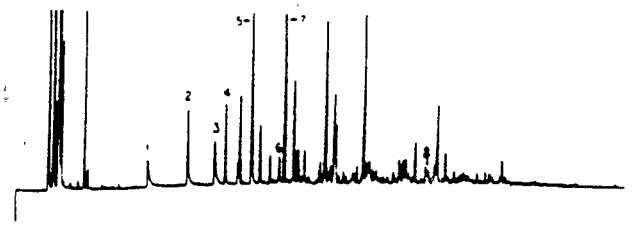

Figure 2. FID chromatogram of basic material isolated from shale oil process water. GC conditions are the same as those given in Figure 1. Compounds identified by GC/MS and confirmed by retention time matching are (1) pyridine, (2) 2-picoline, (3) 4-picoline, (4) 2,6-lutidine, (5) 2,4-lutidine, (6) aniline, (7) 2,4,6-trimethylpyridine, and (8) isoquinoline.

Table III. Compounds Found in Shale Oil Process Water

compound	concn (av of 2 runs), ppm
4-picoline	5.2
2,4-lutidine	17.0
2,6-lutidine	2.9
2,4,6-trimethylpyridine	17.6
aniline	1.6
isoquinoline	1.3

Dichlobenil (chemical name is 2,6-dichlorocyanobenzene) contains only cyano nitrogen. The herbicide Diphenamid (chemical name, *N,N*-dimethyl-2,2-diphenylacetamide) contains only the very weakly basic amide group. The reason for obtaining chromatographic peaks of these three compounds is not entirely clear. Perhaps there is some interaction with the sulfonate group of the cation exchanger or else the solvents used in the washing step failed to remove these compounds from the resin.

A sample of shale oil process water was carried through the ion-exchange concentration scheme with subsequent GC analysis. The chromatogram, shown in Figure 2, contained over 60 peaks. Combined GC/MS analysis indicated that most of the peaks were substituted pyridines, anilines, and quinoline isomers. The identification of several compounds was confirmed by matching retention times with the known compounds. A partial list of the compounds identified is given in Table III. The concentrations were estimated by standard means, using triallylamine as the internal standard.

ACKNOWLEDGMENT

The authors gratefully acknowledge J. Beane, J. Richard, M. Avery, and G. Junk for their help with the GC/MS work and J. Nagy for collection of the Delaware River water sample.

Registry No. Hexylamine, 111-26-2; cyclohexylamine, 108-91-8; octylamine, 111-86-4; 2-ethylhexylamine, 104-75-6; diallylamine, 124-02-7; dipropylamine, 142-84-7; dibutylamine, 111-92-2; dihexylamine, 143-16-8; *N*-methylcyclohexylamine, 100-60-7; dicyclohexylamine, 101-83-7; triallylamine, 102-70-5; tributylamine, 102-82-9; aniline, 62-53-3; *o*-toluidine, 95-53-4; *m*-toluidine, 108-44-1; *o*-chloroaniline, 95-51-2; *p*-chloroaniline, 106-47-8; 2,5-dichloroaniline, 95-82-9; *N*-methylaniline, 100-61-8; *N,N*-dimethylaniline, 121-69-7; *N,N*-diethylaniline, 91-66-7; diphenylamine, 122-39-4; 1-naphthylamine, 134-32-7; *o*-phenylenediamine, 95-54-5; pyrrolidine, 123-75-1; piperidine, 110-89-4; morpholine, 110-91-8; *N*-methylmorpholine, 109-02-4; piperazine, 110-85-0; imidazole, 288-32-4; 3-methylpyrazole, 1453-58-3; 3,5-dimethylpyrazole, 67-51-6; indole, 120-72-9; carbazole, 86-74-8; pyridine, 110-86-1; 2-picoline, 109-06-8; 4-picoline, 108-89-4; 4-isopropylpyridine, 696-30-0; 2,4-lutidine, 108-47-4; 2,6-lutidine, 108-48-5; 2,4,6-trimethylpyridine, 108-75-8; 2-aminopyridine, 504-29-0; 2,2'-bipyridyl, 366-18-7; quinoline, 91-22-5; isoquinoline, 119-65-3; quinaldine, 91-63-4; 2,6-dimethylquinoline, 877-43-0; acridine,

260-94-6; benzo[*h*]quinoline, 230-27-3; benzo[*f*]quinoline, 85-02-9; 2-methylpyrazine, 109-08-0; quinoxaline, 91-19-0; 2,3-dimethylquinoxaline, 2379-55-7; *N,N*-dimethylcyclohexylamine, 98-94-2; acetophenone, 98-86-2; dichlobenil, 1194-65-6; simazine, 122-34-9; atrazine, 1912-24-9; trietazine, 1912-26-1; diphenamid, 957-51-7; water, 7732-18-5.

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RECEIVED for review December 22, 1982. Accepted March 29, 1983. This work was supported by the National Science Foundation under Grant CHE-7906108.

Preconcentration of Trace Elements in Natural Water with Cellulose Piperazinedithiocarboxylate and Determination by Neutron Activation Analysis

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To prepare cellulose piperazinedithiocarboxylate (PID), tosylcellulose was reacted with piperazine to give the aminocellulose which was then reacted with carbon disulfide. The preconcentration of the trace elements was accomplished by the PID column operations. Ten liters of river water or spring water sample was passed through the column at a given pH. The packings were taken out of the column, dried at 110 °C, and then burned to ashes in a low-temperature plasma asher. The ashes were encapsulated in polyethylene rabbits of the pneumatic tube system and subjected to neutron irradiation in a reactor. γ-Ray spectrometry was then performed on the irradiated sample and the concentration of 18 elements in natural water samples was determined.

For simultaneous determination of trace elements in natural water samples, neutron activation analysis has been used effectively because of its high sensitivity. An inherent problem associated with neutron activation of the water samples is finding a preconcentration method by which neutron irradiation and measurement become feasible. Therefore, it is desirable that the concentration of a number of trace elements be made at the same time by a one-step procedure.

To meet these requirements, attempts have been made to synthesize cellulose-based polymers containing the dithiocarboxylate group which forms chelates with a relatively wide variety of metal ions.

Cellulose powder was treated with tosyl chloride to obtain tosylcellulose, and then treated separately with aniline, benzylamine, *n*-butylamine, and piperazine to obtain four aminocelluloses. These aminocelluloses were then treated with carbon disulfide to furnish the corresponding cellulose dithiocarboxylates AND, BZD, BUD, and PID.

Comparative studies have been carried out on these four cellulose dithiocarboxylates in regard to pH dependency of

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Received for review September 12, 1979. Accepted April 25, 1980. The research described herein was supported in part by Grants R803960030 from the U.S. Environmental Protection Agency, Duluth, Minn., EE-77-S-02-4347 from the U.S. Department of Energy, and NSF ENV 74-24276 from the National Science Foundation, and by Contract RP-332-1 with the Electric Power Research Institute.

Covalent Binding of Aromatic Amines to Humates. 1. Reactions with Carbonyls and Quinones

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■ The reaction of several ring-substituted anilines with humate has been studied in aqueous solution. The primary amines bind to humate in two phases. Initially, a rapid, reversible equilibrium is established, which may represent formation of imine linkages with the humate carbonyls. Subsequently, there is a slow reaction that is not readily reversed. The slow reaction is thought to represent 1,4 addition to quinone rings followed by tautomerization and oxidation to give an amino-substituted quinone. The slow reaction is not likely to be imine formation or other processes that require primary amines because *N*-methylaniline, a secondary amine, also reacts with humate. These processes represent ways in which aromatic amines may be converted to latent forms with undetermined effects on the biosphere.

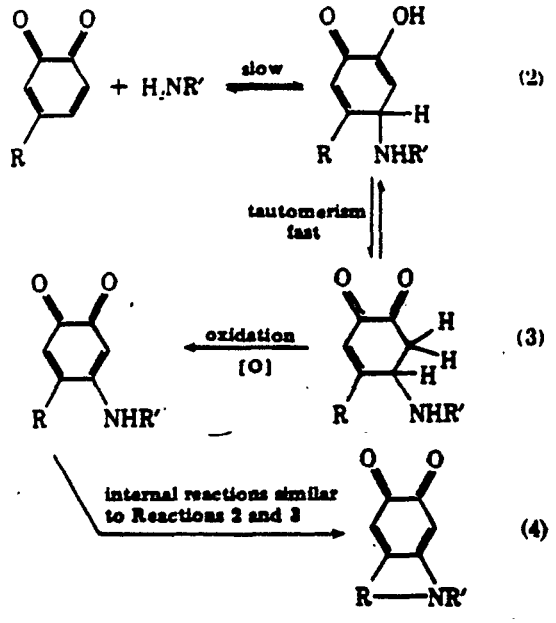
A number of important pesticides, dyes, explosives, and other industrial chemicals contain moieties that are converted to aromatic amines in the environment. Several lines of evidence indicate that aromatic amines become bound to soil organic matter (e.g., humates) by covalent bonds. Important questions have been raised concerning the lability of the amine–humate adduct. In particular, more information is needed about the bioavailability of the aromatic amine moiety, and methods for systematically monitoring these residues are lacking. In this paper, some variables affecting the rate and extent of the reactions between aromatic amines and humates are investigated and discussed in terms of possible modes of adduct formation.

Hsu and Bartha (1, 2) proposed formation of imines (anils, Schiff's bases) to account for the reversible binding of aromatic amines to soil organic matter. Imine formation, Reaction 1, is a well-known reaction (3–5). The reaction is very fast and fully reversible. Water is one of the products of the reaction, and an excess of water tends to drive the equilibrium back to the amine and carbonyl compound. It is likely that amines react with carbonyl groups in humate, but it is not clear where the equilibrium (Reaction 1) lies in aqueous media.

$$ArNH_2 + O{=}CR_2 \rightleftharpoons ArN{=}CR_2 + H_2O \qquad (1)$$

Cranwell and Haworth (6) and later Hsu and Bartha (1, 7) proposed reactions that could lead to the incorporation of primary amines (e.g., aromatic amines and amino acids) into soil organic matter in modes that would be relatively resistant

to hydrolysis and not readily exchangeable. The first step in this process involves a reversible Michael addition to quinonelike structures (present in humic matter due to oxidation of phenols). The addition is followed by tautomerization and oxidation yielding aminoquinones (Reactions 2 and 3). The amino group may react further by essentially the same addition–tautomerization–oxidation sequence to form a variety of nitrogen heterocycles (Reaction 4). The nature of the resulting heterocycle will depend upon the reactive sites (R) in the humate juxtaposed to the aminoquinone.



R = quinone moiety in humate

As a rule of thumb, progression along this sequence of reactions is expected to make the amine moiety more resistant to removal from the humate. After the addition reaction (Reaction 2), the amine residue should not be removed from the humate by simple extraction, but prior to oxidation (and perhaps even after oxidation in some cases) it might be displaced from the humate by a suitable competing reagent. After oxidation (Reaction 3) and particularly after reaction with quinone moieties near the site of the initial addition (Reaction 4), the amine moiety probably resists vigorous acid (6) or base (7) hydrolysis.

It is currently impossible to prove or disprove the existence of these or other linkages of amines to humates by direct spectroscopic means. Indirect support for certain mechanisms can be obtained by selectively blocking humate functional groups before attempting binding experiments. Experiments with compounds that serve as models of humate functional groups demonstrate reactions that might occur with humic matter, but extrapolation of these results to soil organic matter is speculative. With these facts in mind, we report the results of our experiments on the reaction of aromatic amines with humates and compounds that are models of carbonyl and quinone functional groups in humates. The working hypothesis that guided the experiments reported here is that the results pertain to the position and dynamics of Reaction 1 and the rate of Reaction 2.

Experimental

Materials. All chemicals not specifically cited were reagent grade products obtained from commercial dealers. The amines are listed in Table I and abbreviations used in the text are given.

Table I. Gas Chromatographic Retention Times[a]

compound	abbreviation	rel retention time[b]
aniline	A	0.34
N-methylaniline	NMA	0.51
2-toluidine	2MA	0.55
3-toluidine	3MA	0.55
4-toluidine	4MA	0.59
2-chloroaniline	2CA	0.63
4-chloroaniline	4CA	0.84
2-chloronitrobenzene	2CNB	1.00
2,5-dichloroaniline	25DCA	1.49
3,4-dichloroaniline	34DCA	2.22

[a] Perkin-Elmer Model 3920, injector 250 °C, column 150 °C isothermal, detector interface 220 °C; column 6 ft × 4 mm i.d. glass, 10% OV-101 on 80/100 mesh Chromosorb W-HP; helium carrier gas, 33 mL/min. [b] Solvent peak 0.66 min; retention time of 2CNB was 4.74 min.

Humate H-1. Commercially prepared sodium humate (Aldrich Chemical Co., Milwaukee, Wis.) was extracted sequentially with acetone and methanol in a Soxhlet apparatus. Upon evaporation, the acetone extract yielded a light yellow solid (0.01 g/g of humate). The solid obtained from the methanol (0.03 g/g of humate) was brown. The black solid remaining after these extractions was designated H-1. About 30% of H-1 was found to be insoluble in distilled water. The insoluble material appears to be humin.

Humate H-2. Commercial sodium humate was suspended in distilled water (10 g/L) in a shallow bowl. After 5 days the dark liquid was decanted from a gray solid that had settled. The solid obtained by evaporating the dark liquid was extracted with methanol to remove any organic contaminants and designated H-2.

Humate H-3. The commercial product was suspended in 0.5 M sodium hydroxide and centrifuged. The liquid was decanted and a solid was precipitated by adjusting the pH to 2 with HCl. This cycle was repeated three times; the base-soluble solid was then washed with distilled water and freeze-dried. This product, H-3, is an acid rather than a sodium salt, and it does not dissolve readily in water until the pH is raised to about 9. Once dissolved, H-3 will stay in solution at pH 4.

Humate H-RB. Humate H-2 (1.54 g) was treated with 0.21 g of sodium borohydride in 20 mL of pH 9 sodium borate buffer. Foaming was dispersed with 6 mL of 2-propanol. After 1 h, gas evolution was very slow and an additional 0.1 g of $NaBH_4$ was added. The reaction mixture was allowed to stand for 3 days; it was then taken to dryness and methanol was added to destroy any remaining borohydride. A control sample of H-2 was carried through the same procedure excluding addition of $NaBH_4$.

Humate H-RS. Humate H-2 (0.62 g) was treated with 5.0 mL of a dilute HCl solution containing 0.437 g of $SnCl_2$. The solution was mixed by shaking and allowed to stand for 18 h before use in binding experiments. A control sample of H-2 was similarly treated with dilute HCl not containing $SnCl_2$.

Model Compounds. In the course of these studies 3,4-dimethoxybenzaldehyde (veratraldehyde) and 1,4-benzoquinone were used to model simple aldehyde and quinone functional groups that are found in humic matter. Each of these compounds was reacted with 4-chloroaniline on a preparative scale to obtain products that may simulate aniline-humate interactions.

N-(4-Chlorophenyl)-3,4-dimethoxybenzaldehydeimine was prepared by dissolving 1.3 g of 4-chloroaniline and 1.7 g of veratraldehyde in 10 mL of methanol. Within 15 min, the imine precipitated as white needles. The product was re-

crystallized 5 times from methanol, and three crops of white needles were collected (0.2, 1.0, and 0.5 g, respectively). The product's infrared spectrum showed no N—H and no C=O absorptions. The starting aldehyde absorbs at 1660 cm^{-1}. In the product, the 1689–1471-cm^{-1} region where the C=N absorption is expected is obscured by other bands. This product melted at 83–84 °C. The compound did not decompose to 4CA when injected onto the GC-N/P in anhydrous methanol.

The reaction of *p*-benzoquinone with ring-substituted and N-methylanilines in methanol produced deep red solutions. When the quinone was in large excess, the solution was stable for long periods, but when the molar ratio of amine to quinone was 1:1 the red solution rapidly produced a brown precipitate. The amines used in this study did not react with 1,4-hydroquinone in methanol on the time scale at which reaction occurred with 1,4-benzoquinone. However, as 1,4-hydroquinone was oxidized by air to 1,4-benzoquinone the solution did slowly turn red.

The infrared spectra of the red and brown products isolated by column chromatography (benzene/silica gel) from the reaction of 4-chloroaniline with 1,4-benzoquinone in methanol showed only one band for N–H stretching. It is known that primary amines have two N–H bands in their infrared spectra, secondary amines have only one (*8*), and imines ($RN{=}CR_2$) have none; therefore, the products appear to be *N*-(4-chlorophenyl)aminobenzoquinones. In the spectrum of the brown products, the peaks attributable to the 4-chlorophenyl group (1550, 1460, 1380, 1270, 1080, 1000, 810, and 520 cm^{-1}) were more predominant than in the red products.

Buffers. In one set of experiments, commercial aqueous buffers were used as solvents. Unexpected peaks soon appeared in the chromatograms. The commercial buffers contain formalin (aqueous formaldehyde, 0.05%), which reacts with amines to form imines. These imines were identified by mass spectrometry. All the experiments reported here used buffers prepared from reagent chemicals in the laboratory: pH 4 potassium phthalate, pH 7 phosphate, and pH 9 borate. The buffers were rather weak (*e.g.*, 0.05 *m*) and the actual pH of the humate solutions when pH 7 buffer was used was between 7 and 8. For this reason, results refer to the "buffer used" unless it is stated that the pH was measured.

Gas Chromatography. A Perkin-Elmer Model 3920 gas chromatograph with nitrogen–phosphorus detector was used routinely to determine aromatic amines. Good chromatographic separations (Table I) were achieved using a 6 ft × 4 mm i.d. glass column packed with 10% OV-101 on 80/100 mesh Chromosorb W-HP. Helium carrier gas was used at 33 mL/min. The injector temperature was 250 °C and the detector interface was 220 °C. The column was usually run isothermally at 150 °C. However, in a few experiments, 2,4-dichlorotoluene was used as an internal standard with a flame ionization detector, and a lower column temperature, i.e., 115 °C, was required to separate early eluting compounds from the methanol solvent front.

Binding and Release Experiments. Typical binding/release experiments were run in test tubes (10–20 mL) closed with aluminum foil lined screw caps. Humate (0.5–1.0 g) was placed into the tared tube and solvent or buffer was added. The test compounds (usually in mixtures of three–five compounds) were added in methanol solution (1.00–5.00 mL of 1 or 0.1 mg/mL) by pipet. The tubes were shaken as necessary to ensure mixing and stored at ambient temperature (21–23 °C) or kept in a 30 °C bath as noted. When methanol was the solvent, extraction was achieved by repetitive centrifugation and decantation of the liquid. When water was the solvent, the liquid was extracted several times with ethyl acetate. In kinetic experiments with humate, aliquots of the reaction mixture were periodically drawn by pipet, extracted, concentrated, and analyzed.

In the first two preliminary experiments, absolute recoveries were determined using external standards. It soon became apparent that the precision of this method was limited to a standard deviation of ±20% due principally to compounding of errors in volumetric transfers. A systematic error due to incomplete extraction and losses in the drying and concentration steps was also introduced. The latter losses were particularly significant when the extracts were concentrated to 1 mL and they tended to be greater for lower boiling aromatic amines. Thus, for the remaining experiments where higher precision and optimum recoveries were desired, an internal standard, 2-chloronitrobenzene, was added to the amine before it was mixed with the humate or model compounds, and results are reported as recovery relative to the internal standard. By concentrating the extracts with the internal standard only to 5 mL the precision of analysis was limited only by the random variations in detector response and retention times inherent to the GC-N/P system and the measurement of peak heights. The precision was typically ±5% except when peaks less than 10 mm (usually late eluting peaks, i.e., 25DCA and 34DCA) had to be compared with the internal standard. In these cases precision fell to ±10%. The recovery efficiency of all aromatic amines was acceptable, and over a long series of measurements the absolute recovery of the internal standard was found to be 104 ± 21% standard deviation.

Kinetic Experiments with Model Compounds. In kinetic experiments with model compounds, samples of the aqueous methanol reaction mixtures (*e.g.*, 5 μL) were directly injected into the gas chromatograph. Unreacted anilines were determined relative to an internal standard. Linear first-order rate plots, $\ln [A] = -kt + \ln [A]_0$, were obtained for each substituted aniline in the reaction with benzoquinone over more than 90% of the reaction, except for 25DCA and NMA, which were not followed beyond about 30% completion.

Results and Discussion

Characterization of Humates. To ensure that the humate materials used in this study were similar to materials employed in other studies, they were examined by infrared spectroscopy. The spectra were similar to those published by other authors (*9–11*). The humate fractions that had been removed by extraction with organic solvents contained less –OH and more aliphatic C–H than the bulk of the material. The reduced humate H-RB had less absorption in the 1700–1800-cm^{-1} region than H-2, and H-RB had more intense absorption in the 1400–1300- and 1100–1000-cm^{-1} regions. These changes are indicative of reduction of carbonyl functional groups ($\nu_{C=O}$ 1700–1750 cm^{-1}) to alcohols (ν_{C-OH} 1300–1000 cm^{-1}).

Preliminary Experiments. Several preliminary experiments were carried out to determine the effects of certain variables (*e.g.*, nature of the aromatic amine, solvent/pH, ratio of amine to humate, time of reaction, etc.) upon the binding and release of amines by humate. These experiments allowed the development of methodology for more precise kinetic experiments which followed. Since the preliminary experiments were run for arbitrary reaction periods, they do not distinguish between processes that have reached equilibrium and processes that are still approaching equilibrium at some undetermined rate.

Abbreviations for amines (e.g., A = aniline, 4CA = 4-chloroaniline, 25DCA = 2,5-dichloroaniline) are found in Table I.

Competitive Displacement. Fuchsbichler and Suss (*12*) reported that radiolabeled 4CA were extracted from soil more efficiently with solutions of unlabeled 4CA or 34DCA than with pure water or salt solutions. This experiment demonstrates that there must be a limited number of specific binding

sites for which various amines compete. We decided to confirm this observation for our humate systems and use it to estimate the number of exchangeable sites per gram of humate.

A sample of humate H-1 was pretreated with a great excess of 4CA (4.7 mequiv/g of humate) in methanol for 3 days, after which the humate was repeatedly extracted with methanol, washed with CH_2Cl_2, and dried. We anticipated that aniline would be a more reactive amine than 4CA. We added a large amount of aniline (18 μequiv/g of humate) in 1% acetic acid/methanol to the humate and let the mixture equilibrate for 1 day. From the methanol extracts, 5.4 μequiv of 4CA/g of humate was recovered, compared to 1.4 μequiv of 4CA/g of humate recovered in a control without aniline. Since the small amount of aniline could not have significantly affected the gross properties of the solvent, the enhanced recovery with aniline indicates a specific effect. The result also suggests that the humate has at least 4 μequiv of sites for exchangeable binding of primary amine per gram. (There may be more exchangeable and unexchangeable sites.)

Binding and Release of 2CA, 4CA, and 34DCA in Two Solvent Systems. In Table II, a binding and release experiment involving competition of 2CA, 4CA, and 34DCA is summarized. The effects of the amine/humate ratio and solvent are examined. In the binding phase, methanol with either 3% acetic acid or 3% aqueous ammonia (the aqueous ammonia contained 30% NH_3 in water) was used as solvent, and the amines were left in contact with the humate for 3 days. Upon extraction (two 10-mL portions of methanol) and analysis, it was found that the absolute recoveries of all three amines were high in the presence of ammonia (considering that the extracts were concentrated to 1 mL before analysis; see Experimental). In the experiments in which acetic acid was used in the solvent, 4CA was not recovered as efficiently from humate H-1 as 2CA and 34DCA. Also, lower recoveries are experienced when the ratio of amine to humate is lower.

A third 10-mL methanol extract of each sample was taken and analyzed separately to confirm that all free amines had been removed.

In the release phase of the experiment, the humate samples were treated with 3% acetic acid/methanol containing aniline (e.g., 30 μequiv/g of humate). After 3 days the samples were extracted and analyzed. No additional amine was recovered from the humate originally treated with ammonia, but appreciable amounts were recovered from the humate samples originally treated with acetic acid. In particular, 4CA and 34DCA appear to have been selectively sequestered in the binding phase of the experiment.

These experiments demonstrate that there is a reversible (e.g., exchangeable) mode of binding of aniline to humates. The binding sites appear to be saturating at high amine concentrations. Ammonia may be blocking sites by forming imines with available carbonyls or other reactions.

The Ortho Substituent Effect. To determine whether the lack of binding displayed by 2CA relative to 4CA in the preceding experiment was due to steric or electronic effects, parallel binding experiments were run between 2MA and 4CA and between 4MA and 4CA. (This experimental design was chosen because 2MA and 4MA were not resolved in the chromatograms and could not be analyzed simultaneously.) After 5 days in 50% aqueous methanol solution at 30 °C (treatment levels were 8.3 to 11 μequiv of each amine/g of humate H-2), the recoveries of the amines were determined relative to 2CNB internal standards. In both experiments 52% of the 4CA was recovered, but only 34% of the 4MA was recovered while 85% of the 2MA was recovered. These results indicate that ortho substituents inhibit binding of aromatic amines regardless of whether the substituent is electron withdrawing (Cl–) or electron donating (CH_3–).

Blocking Humate Carbonyls. In principle it is possible to deduce which functional groups of humate are responsible for amine binding by selectively blocking them prior to attempting binding experiments (*1*). In practice, this approach is less than ideal because (a) it is impossible to modify one functional group without having effects on the overall properties of the humate, and (b) it is not easy to demonstrate spectroscopically that all the chosen sites were blocked.

Treatment of humate with $SnCl_2$ (*13*) yielded a product that was difficult to manipulate. Tin salts were entrained and the product was not soluble in water. In binding experiments, it behaved much like normal humate.

Reduction of simple carbonyl compounds (*14, 15*) by sodium borohydride has been well established, and this experience

Table II. Binding and Release of Aromatic Amines by Sodium Humate[a]

	solvent system								
	3% AcOH/CH_3OH			3% AcOH/CH_3OH			3% $NH_3(H_2O)/CH_3OH$		
treatment level, μequiv/g of H-1	2-CA 13	4-CA 14	3,4-DCA 11	2-CA 8.86	4-CA 8.87	3,4-DCA 8.79	2-CA 1.8	4-CA 1.1	3,4-DCA 8.86
				(I) Binding Phase[b]					
absolute % recovery[c] after 3 days									
(a) first two methanol extracts	51	24	50	37	7.0	21	63	68	65
(b) third methanol extract	0.5	0.2	0.2	0.02	<0.1	<0.1	0.4	0.8	<1
(c) total	51	24	50	37	7.0	21	63	69	65
Treatment with aniline, μequiv g of H-1, 3% AcOH/CH_3OH	32			26			31		
	2-CA	4-CA	3,4-DCA	2-CA	4-CA	3,4-DCA	2-CA	4-CA	3,4-DCA
				(II) Release Phase[b]					
absolute % recovery[c] after 3 days (two CH_3OH extractions)	1.8	17	12	3.2	4.9	3.7	<0.5	<0.5	<0.5
total recovered[c]	53	41	62	40	13	25	63	69	65

[a] The humate is H-1. [b] Ambient temperature 21–23 °C. [c] Extract concentrated to 1.0 mL.

has been applied to reduction of aldehyde and ketone functional groups in lignin. There are few references to reduction of quinones (*16–18*), but it appears that some quinones can be reduced by this reagent. There do not appear to be any references to reduction of humates with sodium borohydride, but it seems likely that all readily accessible, simple carbonyl functional groups and some "core" functional groups (*19*) can be reduced by an excess of sodium borohydride. Infrared spectra of H-2 and H-RB show some changes indicative of carbonyl reduction by borohydride.

When H-RB and a control of H-2 were treated with A, 2CA, 4CA, 25DCA, and 34DCA in methanol for 3 days, it was observed that recovery of the amines relative to 2CNB was not much better from H-RB than from the unreduced control. However, the recoveries of the amines from the control were so high (e.g., aniline 76% relative to 2CNB) that any difference between H-RB and H-2 would necessarily be small. Previous experiments (Table II) had shown that under basic conditions recoveries of all amines are high. Thus, the humate samples were washed twice with portions of 3% acetic acid/methanol solution and the binding experiment was repeated. This time, enough binding was achieved in the control to make the experiment a valid test. There were significant differences in the recoveries of unbound aniline (54% H-RB, 32% H-2) and 4CA (65% H-RB, 54% H-2) relative to 2CNB. Although more 34DCA was recovered from H-2 (86%) than H-RB (77%), these results are probably within experimental error. Generally, 2CA and 25DCA were recovered nearly quantitatively from both humates (i.e., they do not show significant binding). These results suggest that reduction with sodium borohydride did affect the ability of humate to bind amines, but the effect is not as clear-cut as might be expected if simple carbonyl functional groups were responsible for the bulk of the binding.

Kinetic Experiments with Humate. In spite of the difficulties of interpreting reactions involving complex and ill-defined materials like sodium humate, several kinetic experiments were conducted. The first kinetic runs were set up as competitive reactions in which a mixture of aniline, 2CA, 4CA, 25DCA, and 34DCA (with 2CNB as internal standard) in methanol was added to an aqueous buffer solution of sodium humate (H-2). Aliquots of the liquid were periodically taken for determination of extractable amines. Figure 1 shows a typical set of data in which percent recovery of the amines relative to 2CNB is plotted as a function of time. Binding apparently occurs in two temporal phases.

Phase I Binding. The very first set of data, taken minutes after mixing the reagents, shows small differences in the recoverability of the amines (see, e.g., Figure 1). While the data are not precise enough to reveal sharp distinctions, the trends observed for the initial extraction suggested that (a) all amines were recovered more efficiently when humate was in alkaline buffer than when neutral or acid buffer was used, and (b) even with acid buffers, 25DCA and 2CA were efficiently recovered. All the amines were quantitatively (92–101%) recovered from pH 4 buffer without humate.

Phase II Binding. After the initial binding equilibrium was established (i.e., phase I), the recoveries of aniline, 4CA, and 34DCA continue to decrease slowly (Figure 1). This binding appears to be only partially reversible. For example, in Figure 1, the dashed lines and circles indicate the recoveries of the amines from a sample of the reaction mixture treated at 287 h with excess aniline and extracted at 420 h for comparison to the untreated reaction mixture. Note that the aniline appears to have significantly enhanced the recovery of 4CA, 34DCA, and even 2CA.

In a similar experiment, aniline, 4CA, and 34DCA (30 μequiv each/g of humate) in pH 7 buffer were allowed to react for 195 h at which time recoveries of aniline, 4CA, and 34DCA

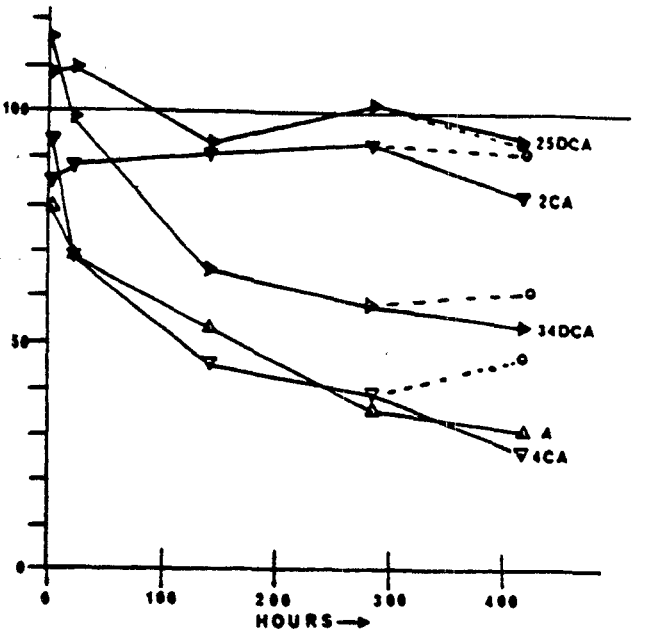


Figure 1. Recovery of substituted anilines from humate in aqueous pH 7 buffer at 30 °C. The initial concentration of humate was 16 g/L (1.64 g of H-2 in 100 mL of pH 7 phosphate buffer to which 3.00 mL of methanol containing the substituted anilines was added). The initial concentrations of the substituted anilines were as follows: A, 4.4×10^{-4} M; 2CA, 2.7×10^{-4} M; 4CA, 3.3×10^{-4} M; 25DCA, 2.1×10^{-4} M; 34DCA, 1.8×10^{-4} M. The data are plotted as percent recovery relative to 100% 2-chloronitrobenzene. The circles indicate recovery of the corresponding amines from an aliquot of the reaction mixture treated with 16×10^{-4} M of aniline at 287 h and extracted at 420 h.

were 48, 43, and 71%, respectively. A large amount of 3MA (3500 μequiv/g of humate) was added as a neat liquid to avoid changing the concentration of the other reactants. After 68 h, recoveries of aniline, 4CA, and 34DCA were 52, 71, and 83%, respectively. However, even after 476 h the recoveries of aniline, 4CA, and 34DCA were still incomplete: 72, 75, and 94%, respectively.

The Kinetic Order of Phase II Binding. Data for the rate of binding of aniline to humate were taken from two of the competitive runs (employing five amines in each) and two other runs in which only aniline and 2CNB were added to the humate in pH 7 buffer. The initial "phase II" rate of binding of aniline, $-d[A]/dt$ at $t = 0$, was determined from plots of concentration vs. time. The results are summarized in Table III and indicate that the rate of binding of aniline varies as the product of the concentrations of aniline and humate.

The reaction thus appears to be first order in aniline and first order in humate. However, this is probably not a simple second-order reaction. If the observed rate were a composite rate, it would still have a second-order relationship as shown by this derivation:

$$-d[A]/dt = k_1[A][S_1] + k_2[A][S_2] \ldots k_n[A][S_n]$$

$$= \sum_i k_i[A][S_i]$$

where S_i are different reactive sites in the humate each with a unique rate constant k_i. When the reaction begins ($t = 0$), $[S_i]_0 = C_i[\text{humate}]_0$, where C_i is a constant characteristic of the humate, so that at $t = 0$;

$$-d[A]/dt = \sum_i (k_i C_i [A]_0 [\text{humate}]_0)$$

$$= (\sum k_i C_i)[A]_0[\text{humate}]_0$$

and $\Sigma_i k_i C_i$ is an appare onstant deter-

Table III. Initial Rate of Phase II Binding of Aniline with Humate in pH 7 Buffer at 30 °C

Initial concentrations: aniline, $\times 10^{-4}$ M	Initial concentrations: humate, g/L	product [A][H], $\times 10^{-4}$ M-g/L	Initial phase II rate $-d[A]/dt$, $\times 10^{-4}$ M/h	2nd-order rate constant, L/(g·h)
14[a]	30	420	0.031	7.38×10^{-5}
5.7	41	234	0.022	9.40×10^{-5}
5.7	26	148	0.019	12.8×10^{-5}
4.4[a]	16	70	0.0058	8.29×10^{-5}
				av $(9.47 \pm 2.05) \times 10^{-5}$

[a] Competitive runs.

mined by dividing the initial rate by the initial concentrations of humate and aromatic amine.

Reactions of Primary Amines with Model Compounds. As reported by Hsu and Bartha (*1*), amines appear to react with humate by two distinct mechanisms. In the absence of methods to directly determine the nature of the chemical bonds between the amines and humate, reactions with model compounds allow the proposed mechanisms to be tested. The results of experiments with model compounds can never prove what is happening, but they can sometimes show what is not happening.

Formation and Decomposition of Imines. Veratraldehyde (3,4-dimethoxybenzaldehyde) was chosen as a humate–model carbonyl. When a mixture of aniline, 2CA, 4CA, 25DCA, 34DCA (e.g., 10^{-3} M), and 2CNB internal standard was mixed with veratraldehyde (10^{-1} M) in 77% CH_3OH/23% H_2O (v/v, pH 5 was due to traces of acid), an equilibrium was established immediately. Direct injection of the reaction mixture into the chromatograph showed loss of free aniline and 4CA within seconds of mixing the aldehyde and amines, and the established concentrations did not change over a period of 5 days. To prove that the imine/aniline equilibrium is rapidly established under these conditions, a sample of the imine formed from 4CA and veratraldehyde was dissolved in methanol and found to be stable indefinitely even when traces of acetic acid were added because a stoichiometric amount of water is required for hydrolysis (Reaction 1). However, when water was added to the solution of the imine (solution diluted to 16% CH_3OH/84% H_2O), free 4CA was immediately liberated. Formation constants (K_f) for imines are usually cited as the equilibrium constant (K_{eq}) for Reaction 1 divided by the concentration of water:

$$K_f = \frac{K_{eq}}{[H_2O]} = \frac{[\text{imine}]}{[\text{amine}][\text{carbonyl}]}$$

Formation constants were calculated for the imines of anilines ($K_f = 9.0\ M^{-1}$) and 4CA ($K_f = 3.6\ M^{-1}$) with veratraldehyde at 22 °C in 77% CH_3OH/23% H_2O, but the formation constants for the other imines were too small to measure at the concentrations used here (e.g., they are less than $1\ M^{-1}$). The thermodynamic equilibrium constants are, of course, much larger since the concentration of water in this solution is about 15 M (e.g., K_{eq} for aniline–imines $9\ M^{-1} \times 15\ M = 135$). These results are similar to those reported by other workers (*3*).

Experiments in which 4CA was displaced from the 4CA imine of veratraldehyde were also conducted. These reactions were run in anhydrous methanol, which made the rates very slow because no more than a catalytic amount of water was present. It was observed that the extent (and rate) of displacement of 4CA by other amines was aniline > 34DCA ≫ 2CA.

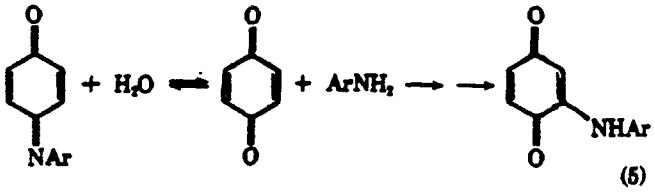
These results are similar to the rapid, reversible (phase I) binding of aromatic amines observed with humate. It is reasonable to propose, as Hsu and Bartha (*1*) did, that imine formation occurs to a significant extent in nonaqueous systems (e.g., anhydrous methanol or dry soil). However, in aqueous systems (e.g., flooded soil) imine formation will tend to be limited by the large excess of water (i.e., $[H_2O]/[ArNH_2] \gg 100$) in spite of favorable equilibrium constants (i.e., $K_{eq} \approx 100$). In other words, we can write:

$$[\text{imine}]/[\text{amine}] = K[\text{humate carbonyl}]_{free}/[H_2O]$$

$$\frac{[\text{bound amine}]}{[\text{free amine}]} = \frac{K([\text{humate carbonyl}]_0 - [\text{imine}])}{[H_2O]}$$

for the rapidly exchangeable, imine-bound anilines where $K = 10$ to 1000 (*3*) and $[\text{humate carbonyl}]_0 = [\text{humate}] \times 10^{-3}$ equiv of carbonyl/g (*20*).

Reactions of Primary Amines with Quinones. Primary amines may reversibly form imines with quinones, but the isolatable products are the less labile quinone substitution products (Reactions 2 and 3).



(5)

For comparison of reactivities of various amines with humate (Figure 1) and quinones, quantitative rate experiments were conducted. Ring-substituted anilines and *N*-methylaniline were allowed to react with *p*-benzoquinone under pseudo-first-order conditions. The results are summarized in Table IV. The rates of reaction are in the order 4MA > aniline > 4CA > 34DCA > NMA ≫ 2CA > 25DCA.

Hammett plots (*21*, *22*) for 4MA, aniline, 4CA, and 34DCA were made using both the usual σ and σ^+ substituent constants (Table IV). The reaction constants ρ, which were calculated from the σ and σ^+ plots, were −1.5 and −1.8, respectively. However, the data are better correlated with the σ^+ constants, which were developed for application to systems in which substituents directly conjugate with an electron-demanding reaction center in the transition state. These results may be compared with $\rho = -0.99$ obtained for the nucleophilic reaction of substituted phenoxides with ethyl iodide in ethanol at 43 °C, and the equilibrium constants for protonation of substituted anilines by formic acid in 67% aqueous pyridine where $\rho = -1.43$ (*22*).

Preparative scale reaction between *p*-benzoquinone and 4CA produced several colored products. The use of high concentrations and quinone/amine ratios approaching 1:1 leads to multiple substitution on the quinoid ring and other side reactions (*23–25*), which were minimized under the conditions of the rate study.

Table IV. Reaction of Substituted Aniline with *p*-Benzoquinone under Competitive Pseudo-First-Order Conditions

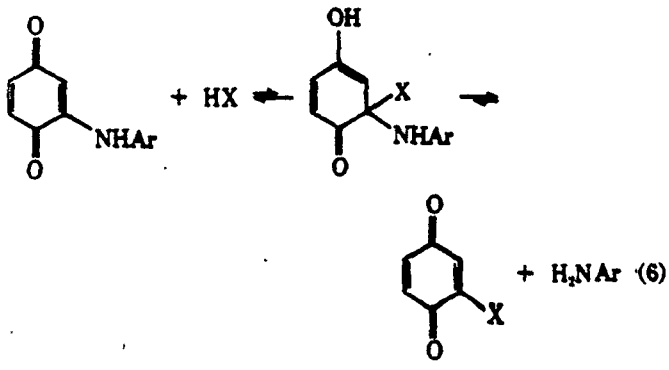
compound	initial[a] concn, M	k_{1_1}, s^{-1}	$k_1/[Q]_i$,[a] $M^{-1}\ s^{-1}$	log (k/k_0)[b]	σ[b]	σ^+[c]
4-methylaniline	0.86×10^{-3}	2.8×10^{-3}	2.9×10^{-2}	0.42	−0.17	−0.26
aniline	1.37×10^{-3}	1.1×10^{-3}	1.1×10^{-2}	0	0	0
4-chloroaniline	1.02×10^{-3}	0.87×10^{-3}	0.90×10^{-2}	−0.087	+0.23	+0.04
3,4-dichloroaniline	0.67×10^{-3}	0.16×10^{-3}	0.17×10^{-2}	−0.81	+0.60	+0.43
2-chloroaniline	0.83×10^{-3}	0.055×10^{-3}	0.057×10^{-2}			
2,5-dichloroaniline	0.57×10^{-3}	0.0062×10^{-3}	0.0064×10^{-2}			
N-methylaniline[d]	0.76×10^{-3}	0.10×10^{-3}	0.12×10^{-2}			

[a] Temperature 22–23 °C, solvent 71% CH_3OH/29% H_2O, initial quinone concentration [Q] = 97×10^{-3} M, except for *N*-methylaniline, where [Q] = 84×10^{-3} M. [b] Log (k/k_0) = $\rho\sigma$, $\rho = -1.50$, $r^2 = 0.954$. [c] Log (k/k_0) = $\rho^+\sigma^+$, $\rho^+ = -1.79$, $r^2 = 0.998$. [d] Reaction carried out similarly to other runs except that flame ionization detection was used with a column temperature of 115 °C, and 2,4-dichlorotoluene (24DCT) was used as an internal standard. Under these conditions the retention time of NMA is 7.6 min and the retention time of 24DCT is 10.8 min. Reaction was followed to 67% completion.

Reaction of a Secondary Amine with Humate and Model Compounds. Inspection of Reactions 1, 2, and 3 suggests that it may be possible to distinguish between some of the possible mechanisms of covalent bond formation by comparing the reactivity of primary $ArNH_2$ and secondary ArNHR amines toward humates and model compounds. Secondary amines, such as NMA, cannot form imines (*3–5*), but as shown in Table IV, NMA reacts with *p*-benzoquinone to form the same sort of red product as the ring-substituted anilines. Note that the rate of reaction of NMA with *p*-benzoquinone is about the same as 34DCA, and the reaction goes to completion given enough time.

In a competitive experiment NMA (5.0×10^{-4} M) and 4CA (6.7×10^{-4} M) were reacted with humate H-2 (19 g/L, measured pH 4 after adjustment with acetic acid and 94% H_2O/6% CH_3OH, v/v). Recoveries of NMA and 4CA after 0.5 h were found to be 97 and 63%, respectively, relative to 2CNB. When the reaction was allowed to proceed for 8 days before extraction and analysis, recoveries of NMA and 4CA were only 60 and 22%, respectively, relative to 2CNB. Recoveries of NMA and 4CA from a sample of humate H-3 prepared by base extraction and reprecipitation of commercial humate (16 g/L, measured pH 4 after adjustment with acetic acid and 94% H_2O/6% CH_3OH, v/v) run in parallel with the H-2 humate experiment for 8 days were 46 and 15% relative to 2CNB.

These results are compatible with Reaction 2 being the rate-limiting step for introduction of the amine moiety into the humate. The use of a secondary amine is a useful tool because it is blocked from forming imines and heterocyclic compounds. If phase II addition does represent addition to quinones by Reaction 2, the partial reversibility observed for phase II is readily explained. Either a simple reversal of Reaction 2 (i.e., retro-Michael reaction (*26*)) or direct substitution as in Reaction 6 can occur (*23, 25*).



(6)

Summary and Conclusions

Experiments indicate that covalent binding (as inferrred from lack of recoverability by simple extraction) of ring-substituted anilines to humates involves two different chemical reactions. When an aniline is mixed with humate, a reversible equilibrium is very rapidly established. This equilibrium is thought to represent reaction of the amino group with aldehyde and ketone groups in the humate to form imine linkages. The second reaction is very slow and not readily reversible. This slow reaction is thought to represent addition of the amines to quinoidal structures followed by oxidation of the product to a nitrogen-substituted quinoid ring. Subsequent to this initial addition, further reactions may occur in loco to lock the amine moiety into the humate as part of a heterocycle. The effects of chloro and methyl substituents on the aniline ring are compatible with these interpretations. In addition, the conclusions are supported by the observation that *N*-methylaniline (which cannot form imines, but which adds to quinoid rings) reacted slowly with humate.

Acknowledgment

The author thanks Pasquale Lombardo and Gregory Diachenko for helpful discussions and criticism of the manuscript. Gregory Diachenko prepared the humate H-3 used in this study.

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Received for review September 10, 1979. Accepted May 2, 1980.

Sorption of Iodide on Copper

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■ The kinetics and thermodynamics of the sorption of iodide on Cu metal, Cu_2O, and CuO have been studied using an ion-selective electrode and a radioactive ^{125}I tracer to measure I^- in solution. ESCA, SEM, and XRD techniques have been used to measure and characterize I^- directly on the copper metal surface. The copper surface consists of hydrated or hydroxylated Cu_2O, but both copper metal and Cu_2O sorb I^- rapidly from solutions having $[I^-] \geq 10^{-6}$ M. Sorption on copper progresses in two steps: formation of a $Cu^+(I^-)(OH^-)$ complex on the surface, followed by crystallization of CuI and release of OH^- into solution. The concentration of I^- in solution (and thus the amount of iodide sorbed at low concentrations) is controlled by the solubility product of CuI ($K_{sp} = 1.1 \times 10^{-12}$). Desorption of iodide from copper metal also is consistent with $[I^-]$ in solution being controlled by the solubility product of CuI. Oxidation of the copper surface to CuO results in much poorer sorption of I^-.

^{129}I is potentially one of the more mobile nuclear fission products because of its long half-life ($t_{1/2} = 1.7 \times 10^7$ years) and tendency to go into solution as an anion that is not retarded by interaction with silicate minerals. As noted very recently by Strachan (*1*), research for removing ^{129}I from nuclear waste has been directed mainly toward the scrubbing processes in reprocessing streams (*2–4*), rather than immobilizing the ^{129}I for long-term storage. Precipitation of ^{129}I as $Ba(IO_3)_2$ (*3*) or PbI_2 (*5*) and incorporation of ^{129}I into sodalite (*1*) have been proposed for long-term storage.

Many countries are now considering multiple containment measures for nuclear waste, which would include sorbent buffers and backfill, as well as stable containers or hosts such as glasses, ceramics, or crystalline solids (*6, 7*). The overall system could include sorbents for isotopes such as ^{129}I, which may be leached from the initial immobilizing medium. Copper metal appeared to us to be an attractive sorber for I for several reasons. First, iodine is known to concentrate around copper deposits (*8*) in nature. Second, a preliminary study of I^- sorption on copper showed that considerable I^- is taken out of solution (*9*). Third, copper has been proposed as an excellent stable container for nuclear waste (*7*). We felt that Cu metal as a container, or a copper salt in the buffer/backfill material, could serve to sorb leached ^{129}I from the waste.

In this work we present detailed results of I^- sorption and desorption on copper metal, Cu_2O, and CuO, using surface-sensitive probes such as electron spectroscopy for chemical analysis (ESCA) (*10, 11*), radiometric tracers, and scanning electron microscopy (SEM) to follow the sorption reaction directly and to elucidate the kinetics, mechanism, and thermodynamics of I^- uptake. The ESCA technique is ideally suited for studying surface reactions on smooth substrates (*12, 13*) like that of sheet copper.

Experimental

The copper metal used was Fisher electrolytic dust, having a grain size of about 30 μm. Fisher reagent grade Cu_2O and CuO and powdered natural cuprous oxide (cuprite) from Ontario were also used. Five grams of the above was added to 50 mL of dilute (10^{-2} to 10^{-6} M) NaI solutions at 22 °C for varying times. Sorbed I^- was determined by measuring the residual I^- in the solution with a pH meter and an Orion specific-ion electrode, having a linear sensitivity down to I^- concentrations of 2×10^{-6} M. The radiometric tracer experiments were carried out at room temperature using 25 mL of NaI solution containing ^{125}I tracer, and 0.25 g of Cu, Cu_2O, or CuO. The analyses were made by β-counting the ^{125}I tracer and comparing the counts in the copper solutions with counts in a similar solution without the copper. Deionized water in Teflon or polyethylene bottles was used throughout.

For the ESCA and SEM observations, polycrystalline copper sheet (purity of at least 99.9%) was cut to 2.1 cm × 1.0 cm plates, degreased in acetone, and then dipped into 5% HNO_3 for 1 min. After the samples were rinsed in water and subsequently dried with acetone, they were suspended in 100 mL of NaI solutions of strengths 8×10^{-6}, 8×10^{-7}, 8×10^{-6}, 8×10^{-5}, and 8×10^{-4} M. At the end of the elapsed periods they were removed from the solution, washed thoroughly in water, and dried by sluicing with acetone before being used for ESCA, SEM, and X-ray diffraction measurements.

Black CuO was grown on the sheet copper plates by enclosing them in a sealed glass tube partially filled with water and heating at 100 °C for 1 week. For sorption experiments, these plates were then suspended in NaI solutions as for copper metal above. A CuI standard for ESCA measurements was prepared by a long-established method (*14*). The sorption capacity of powdered CuO was treated with the I^- sensitive electrode and by radiometric counting.

For desorption studies, Fisher electrolytic granular copper (grain size 100–150 μm) was used instead of the finer dust, since it was easier to filter and transfer. Iodide was first sorbed onto this copper by adding 20 g of Cu to 200 mL of 10^{-4} M NaI and leaving the solution for 72 h. The I^- sorption was followed with the specific-ion electrode. The solution was then decanted, and the copper was washed with water to remove residual I^-. The copper was then added to 100 mL of water at 22 or 82 °C in a stoppered Nalgene bottle, and the desorbed I^- was measured with the specific-ion electrode.

The ESCA spectra were obtained using a McPherson ESCA 36 spectrometer and an [illegible] between the incident photons and [illegible]. Eight

Environ. Sci. Technol. 1983, 17, 72–80

Cross-Linkage between Anilines and Phenolic Humus Constituents

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■ The pesticide degradation intermediates 4-chloroaniline, 3,4-dichloroaniline, and 2,6-diethylaniline were examined for their ability to react with various phenolic humus constituents in the presence of a fungal phenol oxidase. An initial indication of a cross-coupling reaction was obtained by color formation. Although anilines alone were not transformed by a phenol oxidase isolated from the fungus *Rhizoctonia praticola*, they readily cross-linked with phenolic acids or their enzymatic products to form hybrids in the presence of the enzyme. A number of hybrid dimers, trimers, and tetramers were isolated and determined by mass spectrometric analysis. The identities of three hybrid dimers formed from syringic acid coupled with 4-chloroaniline, 3,4-dichloroaniline, or 2,6-diethylaniline and a hybrid tetramer from protocatechuic acid coupled with 2,6-diethylaniline were determined. The formation of the hybrid dimer *N*-(2,6-diethylphenyl)-2,6-dimethoxy-*p*-benzoquinone imine from a cross-linkage between syringic acid and 2,6-diethylaniline was quantitatively evaluated.

Introduction

Extensive investigations of the fate of pesticides and other xenobiotics in soil have been performed, but the site and mechanism of their binding to or incorporation into soil constituents are still undefined. One group of chemicals that undergo little transformation or mineralization in the soil within a growing season are the halogen- or alkyl-substituted anilines, which represent the aromatic base of a great number of currently used pesticides (*1*, *2*). While many substituted aromatic pesticides are subject to microbial transformation, the aniline rings are quite resistant and are easily bound to humic material (*1*–*6*).

Hsu and Bartha (*4*, *7*) using ^{14}C-labeled 3,4-dichloroaniline found that about half of the humus-bound anilines could be released by hydrolysis, while the remainder was liberated only by combustion. This led to their conclusion that at least two different mechanisms of covalent binding to soil organic matter exist: the weaker chemical bonds could be condensations of primary amino groups to carbonyl groups forming anils (imines), and the stronger bonds could be explained by the formation of anilinoquinones or the incorporation of the amino groups into heterocyclic ring structures. Similar assumptions were made by Parris (*8*) in studies on the kinetics of the proposed reactions.

While the covalent bonding of aromatic amines to soil organic matter could only be inferred from observations in previous studies, it was our purpose to identify the chemical products formed by reactions occurring between the xenobiotic anilines and constituents of humic material. Our experiments were performed in an aqueous medium and initiated by a fungal phenol oxidase.

Materials and Methods

Materials. An extracellular [illegible] *culture filtrate of Rhizoctonia praticola* was used (*9*). One unit of this enzyme is defined as that amount which causes a change in optical density of 1.0/min at 468 μm in 3.[illegible] of 0.1 M phosphate buffer solution (pH 6.9) containing 3.24 μmol of 2,6-dimethoxyphenol at 23 °C.

4-Chloroaniline, 3,4-dichloroaniline, and 2,6-diethylaniline were obtained from Aldrich Chemical Co. (Milwaukee, WI). The two chloroanilines were treated with activated charcoal and recrystallized twice from petroleum ether. 2,6-Diethylaniline was purified by thin-layer chromatography using Silica Gel F-254 with hexane:ethyl acetate (17:3 v/v) as a development solvent. Radiolabeled 2,6-diethyl[U-^{14}C]aniline hydrochloride with a specific activity of 11.4 mCi/mmol was purchased from New England Nuclear, Boston, MA, and 0.005 μCi (11 100 dpm) of radioactivity was added to 1 mL of the incubation mixture. Vanillic acid, α-resorcylic acid, and β-resorcylic acid were purchased from Aldrich Chemical Co. (Milwaukee, WI), salicylic acid from Fisher Scientific Co., and ferulic acid, caffeic acid, syringic acid, protocatechuic acid, gallic acid, phloroglucinol, resorcinol, and orcinol from Tridom Chemical Inc., New York. The chemical purity of all reagents was determined by chromatographic analysis (TLC and HPLC).

Substrate Incubation and Product Extraction. The phenols and anilines were dissolved in ethanol and added to 0.1 M phosphate buffer solution (pH 6.9) containing 0.5 units of enzyme/mL of solution. If not stated otherwise, the concentrations of phenol and aniline were each 150 μg/mL, and the ethanol concentration did not exceed 0.4%. For preliminary experiments and for determination of color reactions, 10 mL of incubation mixture were used, and 100–200 mL—depending on product yield—were used for the isolation of products.

After 1 h of incubation at 30 °C, the reaction mixture was extracted with an equal volume of diethyl ether. The organic phase was treated with anhydrous sodium sulfate and subsequently evaporated to dryness with a rotary evaporator. The residues obtained were redissolved in an aliquot of methylene chloride and used for thin-layer (TLC) and high-performance liquid (HPLC) chromatography analysis. An assay mixture of boiled enzyme was always included as a control.

For the quantitative experiments of the hybrid dimer produced between syringic acid and 2,6-diethylaniline, the reaction was carried out in phosphate buffer solution (0.1 M, pH 6.9) containing 0.5 units of enzyme, syringic acid ranging from 125 to 750 μg, and 2,6-diethylaniline from 125 to 1000 μg/mL of solution. In a separate experiment, the concentrations of syringic acid and 2,6-diethylaniline were 500 μg and the enzyme concentration ranged from 0.031 to 3 units/mL of reaction mixture. For each treatment, a 20-mL volume of reaction mixture was used. For the experiment with different substrate concentrations, the enzyme assays were incubated at 30 °C for 1 h, and for the experiment with different enzyme concentrations at 30 °C for 20 and 90 min. The reaction mixtures were extracted twice with equal volumes of diethyl ether, and the combined extracts were evaporated to dryness under a stream of nitrogen.

Analytical Methods. TLC was carried out on silica gel F-254 plates (Brinkmann Instruments, Inc., Westbury,

Table I. Color of Reaction Mixture of Various Combinations of Phenols and Anilines after Incubation with a Laccase from the Fungus *R. praticola*

phenolic substrate	control	4-chloroaniline	3,4-dichloroaniline	2,6-diethylaniline
hydroxycinnamic acid				
ferulic acid	light pinkish yellow	reddish yellow	reddish yellow	reddish yellow
caffeic acid	light pink	reddish pink	reddish pink	reddish pink
hydroxybenzoic acid				
vanillic acid	yellow	dark brown	dark brown	dark brown
syringic acid	light reddish brown	dark brown	dark brown	red (ppt)
protocatechuic acid	yellow brown	dark purple	dark purple	dark purple
gallic acid	yellow	dark green brown	dark green brown	dark green brown
α-resorcylic acid	yellow	yellow brown	yellow brown	yellow brown
β-resorcylic acid	light yellow	light yellow	light yellow	light yellow
salicylic acid	no color	no color	no color	no color
hydroxybenzene				
phloroglucinol	light yellow	yellow	yellow	yellow
resorcinol	yellow	pinkish brown	pinkish brown	pinkish brown
hydroxytoluene				
orcinol	yellow	brown	brown	brown

NY) with a layer thickness of 0.25 mm for routine analysis and 0.5 mm for the isolation of products. A solvent system of chloroform–acetic acid (20:1 v/v) was used for most of the TLC analyses. In some cases a second solvent system consisting of hexane:ethyl acetate (8:2 v/v) was employed. All products were extracted from thin-layer plates with ethyl acetate.

HPLC was performed with two Model 6000A pumps, a U6K injector, and 440 UV detector with dual wavelength (254 and 280 nm; Waters Associates, Inc., Milford, MA). The separation was achieved by a Radial-Pak Cartridge (10 μm) by using a radial compression module (RCM-100). Samples were eluted with hexane:ethyl acetate (6:4 v/v) at a flow rate of 1.5 mL/min. All samples were passed through a millipore filter (0.22 μm) before injection into HPLC.

For quantitative analysis, the dried residue was dissolved in 6 mL of methanol, and samples of 20 μL were injected into the HPLC. Triplicate samples were evaluated for each treatment. The yield of the hybrid dimer (m/z 299) was determined by a data module (Waters Associates, Inc., Milford, MA) by using the external standard method. The calibration curve of the dimer was linear in the range between 0 and 25 μg.

Mass spectrometry was performed with electron impact analysis at 70 eV with sample introduction by direct insertion probe on an AEI MS-902 or Kratos MS-50 mass spectrometer at tempertures varying from 300 to 400 °C. Proton NMR spectra were taken on a Bruker 200 MHz instrument with a Fourier transform system using deuteriochloroform as a solvent.

UV spectra were measured in methanol with a Bausch and Lomb 2000 spectrophotometer. Radioactivity was measured with an Isocap-300 liquid scintillation counter (Searle Co.) with samples prepared in a Scinti Verse cocktail (Fisher Scientific Co.). Melting points were obtained in open capillaries in an electrothermal melting point apparatus and are uncorrected.

Acetylation of Product. Reductive acetylation was carried out according to the method of Vogel (*10*). The sample (50 mg) was dissolved in 5 mL of acetic anhydride, and 300 mg of sodium acetate and 300 mg of zinc dust were added. The mixture was gently warmed until the color of the quinone disappeared and then boiled under reflux for 2 min. After the addition of 2 mL of glacial acetic acid, the mixture was boiled again to dissolve the product and the precipitated zinc acetate. The hot solution was poured into 200 mL of cold water, causing hydrolysis of the acetic product was then recovered by methylene chloride extraction.

Results

Previously it was shown that the extracellular fungal laccase of *R. praticola* did not transform chlorinated and brominated anilines (*11*), and this was confirmed with 4-chloroaniline and also observed with 3,4- dichloroaniline and an alkylated derivative, namely 2,6-diethylaniline. Analysis of the enzyme reaction mixtures of the various anilines by TLC did not indicate disappearance of the substrates. Since various anilines reacted with 2,4-dichlorophenol in the presence of the enzyme and produced a particular color (*12*), it was assumed that the formation of a specific color was indicative of cross-coupling products generated during the incubation of naturally occurring phenols with the three anilines. Incubation of 4-chloroaniline, 3,4-dichloroaniline, or 2,6-diethylaniline together with the fungal enzyme and a phenolic humus derivative resulted in most cases in the formation of a color that differed from the color of the reaction mixture containing only phenol; only β-resorcylic acid and salicylic acid did not show a color change (Table I).

The color difference served as an indicator of a cross-coupling reaction between anilines and phenolic humus constituents. Ether extracts of various assay mixtures of anilines and phenols were first analyzed by TLC, and it was usually possible to distinguish some characteristic colored zones that were not observed when phenol alone was the substrate. The colored zone with a specific R_f value on a thin-layer plate occasionally represented a single product, but in most cases it was composed of several products as further analysis by HPLC revealed. The latter technique also provided purification which facilitated chemical identification.

It has to be emphasized that the following description of cross-coupling products between the three anilines and the various phenolic humus constituents covered only the products that were clearly determined under the experimental conditions. Changes in incubation time or the use of different amounts of enzyme units would have resulted in additional or other products. Prolonged incubation or higher enzyme concentration caused further polymerization reaction, which was not of interest since we intended to determine the initial cross-coupling substances which usually undergo further oxidative coupling.

Hybrid Products of 4-Chloroaniline and Humus Constituents. Table II pr
4-chloroaniline and four phenolic

Table II. Hybrid Products of 4-Chloroaniline and Various Phenolic Humus Constituents Formed after Incubation with a Laccase from *R. praticola*

reaction of 4-chloroaniline with:	molecular ion of hybrid product, *m/z*	oligomer	suggested composition[a]	R_f in TLC[b]	color of spot on TLC plate
vanillic acid	371	trimer	$2V + CA - 2CO_2$	0.68	light brown
	374	trimer	$V + 2CA - CO_2$	0.48	gray
	413	trimer	$2V + CA - CO_2 - 2H$	0.77	yellow
	415	trimer	$2V + CA - CO_2$	0.77	yellow
	429	trimer	2V[c] + CA	0.34	orange
	499	tetramer	$V + 3CA - CO_2$	0.34	orange
	506	tetramer	$2V + 2CA - CO_2 - C$	0.64	yellow-green
	540	tetramer	$2V + 2CA - CO_2$	0.34	orange
	554	tetramer	2V[c] + 2CA	0.34	orange
syringic acid	277	dimer	$Sy + CA - CO_2 - 2H$	0.68	yellow
	279	dimer	$Sy + CA - CO_2$	0.68	colorless
	323	dimer	Sy + CA	0.41	yellow
protocatechuic acid	235	dimer	$Prot + CA - CO_2$	0.50	brown
	343	trimer	$2Prot + CA - 2CO_2$	0.28	brown
	431	trimer	2Prot + CA	0.28	brown
	467	tetramer	$Prot + 3CA - CO_2$	0.85	green-brown
	468	tetramer	$2Prot + 2CA - 2CO_2$	0.85	green-brown
gallic acid	419	trimer	$2\,Gal + CA - CO_2$	0.15	brown

[a] CA = 4-chloroaniline, V = vanillic acid, Sy = syringic acid, Prot = protocatechuic acid, Gal = gallic acid. [b] Solvent: chloroform:acetic acid (20:1 v/v). [c] $C_{15}H_{15}O_5$ (*m/z* 304; dimer of vanillic acid and methoxy-*p*-quinone; see ref 14).

humus constituents. A variety of hybrid trimers and tetramers were isolated when vanillic acid was incubated with 4-chloroaniline, but no dimeric hybrids could be detected. The majority of trimers consisted of one aniline and two vanillic acid molecules, and the tetramers consisted of two aniline and two vanillic acid molecules. Only one trimer was found to be composed of one vanillic acid and two 4-chloroaniline molecules, and one tetramer consisting of one vanillic acid and three 4-chloroaniline molecules was characterized.

Three hybrid dimers were formed during coupling reactions of syringic acid and 4-chloroaniline. A yellow hybrid dimer with *m/z* 277 was produced in large yields; the compound had a UV absorbance maximum at 308.7 nm. It could be isolated by HPLC, its molecular weight was determined by mass spectrometry (Figure 1A), and its structural assignment was elaborated by NMR analysis (Table III, compound A). The correct chemical shift assignments for the H_c and H_d protons is based on NMR studies by Iwan et al. (*13*) in similar systems. Therefore, compound A was identified as *N*-(4-chlorophenyl)-2,6-dimethoxy-*p*-benzoquinone imine (Figure 2A). The reduced form of the dimer was determined by high-resolution mass spectral measurement and indicated an elemental composition of $C_{14}H_{14}O_3NCl$ (calculated 279.0659, found 279.0649).

Among the five hybrid products formed between 4-chloroaniline and protocatechuic acid, one compound was produced in large quantity and appeared to be stable. It was isolated by TLC and found to be a tetramer with *m/z* 468 (Figure 3). Another tetramer with *m/z* 467 was composed of three aniline molecules and one protocatechuic acid and had an elemental composition of $C_{24}H_{18}N_3OCl_2$ (calculated 467.0359, found 467.0331) as determined by high-resolution mass spectrometry.

Only one hybrid trimer was obtained between gallic acid and 4-chloroaniline.

Hybrid Products of 3,4-Dichloroaniline and Phenolic Humus Constituents. The cross-coupling products obtained from 3,4-dichloroaniline and various phenolic acids are given in Table IV. Most of the hybrid products obtained during the coupling reaction of vanillic acid and 3,4-dichloroaniline are analogous in structure to those from vanillic acid and 4-chloroaniline. Various types of vanillic acid dimers (*14*) were found to couple with 3,4-dichloroaniline.

Three hybrid dimers and one hybrid tetramer were obtained in the coupling reaction of syringic acid and 3,4-dichloroaniline. A yellow hybrid dimer with *m/z* 31[illegible] produced in high yield and was purified by HPLC[illegible] compound has a UV absorbance maximum at 206[illegible] NMR analysis of this compound allowed the assignment of structure B—as shown in Table III—by analogy with the NMR of compound A, and accordingly compound B was identified as *N*-(3,4-dichlorophenyl)-2,6-dimethoxy-*p*-benzoquinone imine (Figure 2B). The reduced form of the dimer had an elemental composition of $C_{14}H_{13}O_3NCl_2$ (calculated 313.0270, found 313.0250) as shown by high-resolution mass spectral measurement. This compound is analogous to the hybrid dimer with *m/z* 279 formed between syringic acid and 4-chloroaniline (Figure 2). The mass spectra of the hybrid dimer (*m/z* 311) and its reduced form (*m/z* 313) are shown in Figure 1B.

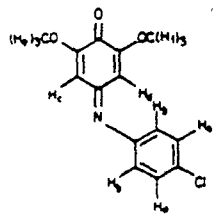
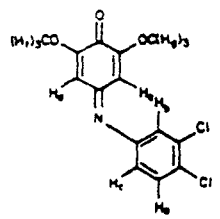
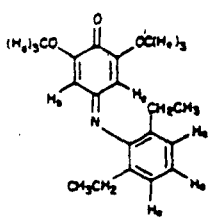
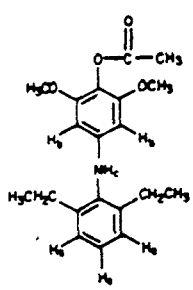
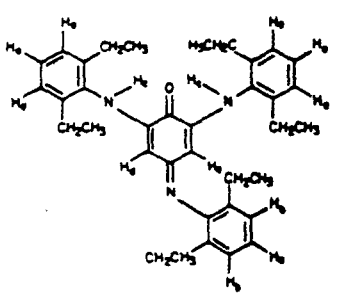
Three hybrid products were detected in a reaction between protocatechuic acid and 3,4-dichloroaniline. A hybrid tetramer with *m/z* 569 was similar in structure to the hybrid tetramer (*m/z* 467) obtained between protocatechuic acid and 4-chloroaniline, but it contained three additional chlorines.

Cross-coupling of ferulic acid and 3,4-dichloroaniline resulted in the formation of three hybrid trimers and one hybrid tetramer.

Hybrid Products of 2,6-Diethylaniline and Phenolic Humus Constituents. A clear indication that cross-coupling between [U-^{14}C]-2,6-diethylaniline and phenolic acids had taken place was obtained by determining radioactivity in the resulting products. In each reaction mixture several radioactive compounds were fou[illegible]nd the hybrid products isolated and determined [illegible]s spectrometry are listed in Table V. The results conc[illegible]g the effect of substrates and enzyme concentration on the hybrid formation will be later described.

A hybrid dimer between syringic acid and 2,6-diethylaniline with *m/z* 299 (Figure 2C) appears to be a major product, since prolonged incubation resulted in the accumulation of this product and the formation of other un-

Table III. Proton NMR Data for Five Oxidative Coupling Products of Phenols and Anilines

compound	chem shift, ppm	splitting (integral)[a]	proton assignments	coupling Hz
	7.377	d (2 H)	H_a	8.8
	6.839	d (2 H)	H_b	8.8
	6.421	d (1 H)	H_c	2.1
	6.054	d (1 H)	H_d	2.1
	3.88	s (1 H)	H_e	
	3.68	s (1 H)	H_f	
A				
	7.473	d (1 H)	H_a	8.5
	7.048	s (1 H)	H_b	
	6.771	d (1 H)	H_c	8.5
	6.456	s (1 H)	H_d	
	6.002	s (1 H)	H_e	
	3.90	s (3 H)	H_f	
	3.72	s (3 H)	H_g	
B				
	7.024-7.085	m (3 H)	H_a	
	6.524	d (1 H)	H_b	2.1
	5.711	d (1 H)	H_c	2.1
	3.891	s (3 H)	H_d	
	3.506	s (3 H)	H_e	
	2.33	q (4 H)	Et CH_2	7.5
	1.05	t (6 H)	Et CH_3	7.5
C				
	7.16-7.30	m (3 H)	H_a	
	5.727	s (2 H)	H_b	
	5.149	br s (1 H)	H_c	
	3.673	s (6 H)	methoxy	
	2.601	q (4 H)	Et CH_2	7.6
	2.301	s (3 H)	acetate CH_3	
	1.166	t (6 H)	Et CH_3	7.6
D				
	7.182-7.329	m (7 H)	H_a	
	7.058	d (1 H)	H_b	7.6
	5.25-5.40	br s (2 H)	H_c	
	5.207	s (1 H)	H_d	
	4.547	s (1 H)	H_e	
	2.16-2.75	m (12 H)	Et CH_2	7.7
	1.318, 1.259, 1.185, 1.072	t (18 H)	Et CH_3	7.7
E				

[a] s = singlet; d = doublet; t = triplet; q = quartet; m = multiplet; br = broad.

identified products was negligible. The compound purified by HPLC had a red color, a melting point of 135 °C, and a UV absorbance maximum at 205.3 nm. The mass spectra of the hybrid dimer (m/z 299) and its reduced form (m/z 301) are represented in Figure 1C; the high-resolution mass spectral data of the quinone dimer (m/z 299) are listed in Table II.

The reductively acetylated product of the hybrid dimer (m/z 299) gave a colorless monoacetate with m/z 343. High-resolution mass spectroscopy gave an elemental composition of $C_{20}H_{25}NO_4$ (calculated 343.1784, found 343.1775). The loss of one ketene gave an ion at M^+ 301 with an elemental composition of $C_{18}H_{21}NO_3$ (calculated 301.1668, found 301.1667). The NMR data in Table III allow the assignment of structure C to the dimer M^+ 299 and structure D to the reductive acetylation product of compound C. Compound C could be identified as *N*-(2,6-diethylphenyl)-2,6-dimethoxy-*p*-benzoquinone imine (Figure 2C).

Incubation of protocatechuic acid and 2,6-diethylaniline resulted in the formation of an orange tetramer, which was purified by HPLC. It consisted of one protocatechuic acid

Table IV. Hybrid Products of 3,4-Dichloroaniline and Phenolic Humus Constituents Formed after Incubation with a Laccase from *R. praticola*

reaction of 3,4-dichloroaniline with:	molecular ion of hybrid product, *m/z*	oligomer	suggested composition[a]	R_f in TLC[b]	color of spot on TLC plate
vanillic acid	403	trimer	2V + DCA − 2CO$_2$ − 2H	0.41	yellow
	447	trimer	2V + DCA − CO$_2$ − 2H	0.41	yellow
	449	trimer	2V + DCA − CO$_2$	0.41	yellow
	463	trimer	2V[c] + DCA	0.41	yellow
	560	tetramer	2V + 2DCA − 2CO$_2$ − 4H	0.35	orange
	608	tetramer	2V + 2DCA − CO$_2$	0.35	orange
syringic acid	311	dimer	Sy + DCA − CO$_2$	0.65	yellow
	313	dimer	Sy + DCA − CO$_2$	0.65	colorless
	357	dimer	Sy + DCA	0.71	yellow
	617	tetramer	3Sy + DCA − 3CO$_2$	0.71	yellow
protocatechuic acid	377	trimer	2Prot + DCA − 2CO$_2$	0.56	gray
	428	trimer	Prot + 2DCA − CO$_2$	0.56	gray
	569	tetramer	Prot + 3DCA − CO$_2$	0.84	green brown
ferulic acid	501	trimer	2Fer + DCA − CO$_2$	0.50	yellow
	512	trimer	Fer + 2DCA	0.50	yellow
	545	trimer	2Fer + DCA	0.60	light pink
	603	tetramer	3Fer + DCA − 3CO$_2$ − 2H	0.93	yellow green

[a] DCA = 3,4-dichloroaniline, V = vanillic acid, Sy = syringic acid, Prot = protocatechuic acid, Fer = ferulic acid. [b] Solvent: chloroform:acetic acid (20:1 v/v). [c] $C_{17}H_{12}O_5$ (*m/z* 304; dimer of vanillic acid and methoxy-*p*-quinone; see ref 14).

Table V. Hybrid Products of [U-^{14}C]-2,6-diethylaniline and Phenolic Humus Constituents Formed after Incubation with a Laccase from *R. praticola*

reaction of 2,6-diethylaniline with:	molecular ion of hybrid product,[a] *m/z*	oligomer	suggested composition[b]	R_f in TLC[c]	color of spot on TLC plate
vanillic acid	418	trimer	V + 2DEA − CO$_2$	0.85	gray
syringic acid	299	dimer	Sy + DEA − CO$_2$	0.84	red
	301	dimer	Sy + DEA − CO$_2$ − 2H	0.84	colorless
protocatechuic acid	517	tetramer	3Prot + DEA − 2CO$_2$	0.80	orange
	533	tetramer	Prot + 3DEA − CO$_2$	0.76[d]	orange
ferulic acid	445	trimer	2Fer + DEA − 2CO$_2$	0.53	yellow

[a] All products contained radioactivity. [b] DEA = 2,6-diethylaniline, V = vanillic acid, Sy = syringic acid, Prot = protocatechuic acid, Fer = ferulic acid. [c] Solvent: chloroform:acetic acid (20:1 v/v). [d] Solvent: hexane:ethyl acetate (8:2 v/v).

Table VI. High Resolution Mass Spectral Data of a Hybrid Dimer (M^+ 299) Resulting from Cross-Linkage of Syringic Acid and 2,6-Diethylaniline

m/z meas'd	*m/z* calcd	possible composition	possible fragment	intensity, %
299.1523	299.1521	$C_{18}H_{21}NO_3$		100
284.1289	284.1287	$C_{17}H_{18}NO_3$	$M^+ - CH_3$	74.76
268.1345	268.1338	$C_{17}H_{18}NO_2$	$M^+ - CH_3O$	20.31
256.1331	256.1338	$C_{16}H_{18}NO_2$	$M^+ - C_2H_5O$	62.69
240.1142	240.1151	$C_{16}H_{14}O_2$	$M^+ - C_2H_5NO$	21.59
226.0951	226.0955	$C_{15}H_{14}O_2$	$M^+ - C_3H_7NO$	8.66

and three 2,6-diethylaniline molecules, and high-resolution mass measurement of the tetramer yielded an elemental composition of $C_{36}H_{43}N_3O$ (calculated 533.3406, found 533.3390) (Figure 4). The prominent peaks at *m/z* 518 and *m/z* 504 corresponded to the loss of CH_3 resulting in an elemental composition of $C_{35}H_{40}N_3O$ (calculated 518.3172, found 518.3178) and the loss of C_2H_5 resulting in an elemental composition of $C_{34}H_{38}N_3O$ (calculated 504.3001, found 504.3008), respectively. The NMR spectrum of this compound allows the assignment of structure E to this tetramer (Table III).

Effects of Enzyme and Substrate Concentration on the Formation of the Hybrid Dimer *N*-(2,6-Diethylphenyl)-2,6-dimethoxy-*p*-benzoquinone Imine (*m/z* 299) Formed from Syringic Acid and 2,6-Diethylaniline. Previously we have shown that syringic acid alone can be polymerized by a laccase, and various oligomers ranging from dimers to hexamers have been produced (*15*). In this study, we noted that in the presence of the laccase most of the syringic acid was coupled with 2,6-diethylaniline through an imine linkage to form a hybrid dimer, while little syringic acid was polymerized to higher oligomers.

Table VII. Effect of Enzyme Concentration on the Formation of Hybrid Dimer (*m/z* 299) of Syringic Acid and 2,6-Diethylaniline

concn of enzyme, units/mL	hybrid dimer *m/z* 299, μg/mL[a] 20 min	90 min	significance of differences between times
0.031	19.6 ± 1.6[a]	119.4 ± 11.6[a]	$P < 0.05$
0.063	42.3 ± 3.6[ab]	197.3 ± 14.7[b]	$P < 0.01$
0.125	84.8 ± 8.2[b]	364.5 ± 25.6[c]	$P < 0.01$
0.25	203.7 ± 14.4[c]	530.8 ± 24.3[d]	$P < 0.01$
0.5	388.6 ± 24.8[d]	728.9 ± 37.4[e]	$P < 0.01$
1	577.8 ± 29.9[e]	863.3 ± 48.2[f]	$P < 0.01$
2	776.9 ± 43.0[f]	864.0 ± 47.9[f]	not significant
3	866.1 ± 50.8[g]	862.1 ± 38.4[f]	not significant

[a] Values in the column for a given time labeled with the same letter are not significantly different at 0.05 level by Duncan's multiple range test. Each value is the mean ± SE of three replicates.

The concentrations of the enzyme and the two substrates were important factors affecting the rate of hybrid dimer formation. There was a clear correlation between

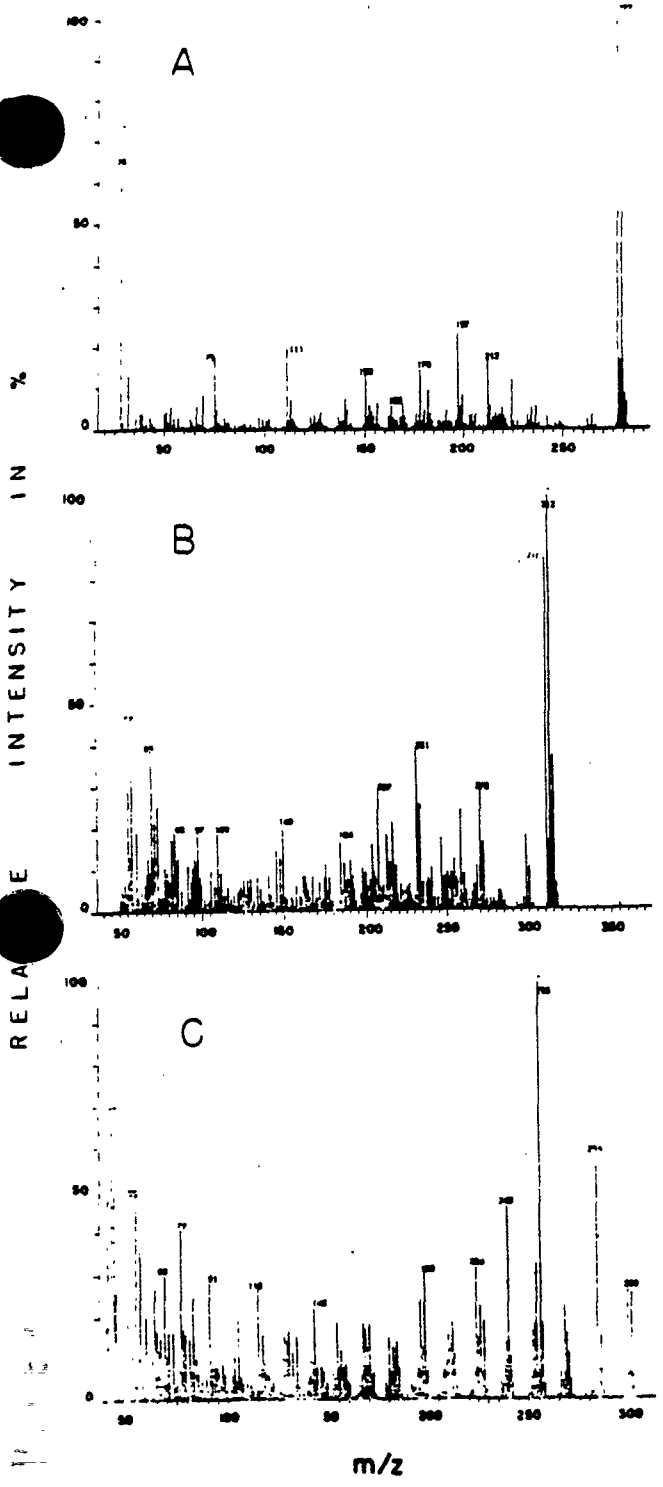


Figure 1. Mass spectra of hybrid products between syringic acid and 4-chloroaniline (A), 3,4-dichloroaniline (B), and 2,6-diethylaniline (C).

enzyme concentration and yield of the dimer (Table VII). When the dimer was incubated for 20 min, its yield differed significantly at enzyme concentrations ranging from 0.125 to 3 units/mL, but no significant differences were observed between 0.031 and 0.063 units/mL or between [illegible] and 0.125 units/mL; maximum yield was obtained [illegible] enzyme concentration of 3 units/mL.

Prolonged incubation of 90 min reduced the differences in hybrid dimer production at various enzyme levels. The yields of the dimer were significantly different at enzyme concentrations ranging form 0.031 to 1 unit/mL, and maximum yield was obtained at an enzyme concentration of 1 unit/mL.

There were significant differences in dimer production at the two incubation times with enzyme concentrations below 1 unit/mL but no differences at higher enzyme levels. The yield of the dimer was about 20 μg/mL at the lowest enzyme concentration (0.031 units/mL); extending the incubation time from 20 to 90 min resulted in a 6-fold increase in dimer production. At a concentration of 0.5 units/mL, which is used for routine assays, extended incubation time brought about a 2-fold increase in dimer production, amounting to 85% of the maximum yield at higher enzyme levels.

It is apparent that maximum dimer yield was dependent on the combination of concentrations of the two substrates (Figure 5). The yield of hybrid dimer increased with increasing syringic acid concentration. Syringic acid above 500 μg/mL combined with higher concentrations of 2,6-diethylaniline resulted in a decrease of hybrid dimer formation. The dimer yields at syringic acid concentrations of 125, 250, and 500 μg/mL were significantly different at $p < 0.05$, but no differences were observed at concentrations between 500 and 750 μg/mL. Hybrid dimer formation increased with increasing amounts of 2,6-diethylaniline (up to 375 μg/mL in the presence of 125 and 250 μg/mL of syringic acid), and it decreased gradually at higher 2,6-diethylaniline concentrations. If the aniline concentration was higher than 500 μg/mL, an inhibitory effect on the production of the hybrid dimer was observed, and this phenomenon became more evident with increasing syringic acid concentrations. Maximum hybrid dimer formation occurred when concentrations of both syringic acid and 2,6-diethylaniline were 500 μg/mL.

Discussion

The binding of pesticides to humus is very difficult to characterize since humus material is a heterogeneous and complex entity. However, the characterization of chemical bonding of pesticides to humus can be approached through models that allow the determination of accurate molecular configurations (*16*). Various indications of chemical binding of pesticides to humus have been suggested by Stevenson (*17*), and various reports have been recently reviewed by Bartha (*1*) and Parris (*18*).

In this paper we attempted to work out the chemical reactions initiated by a fungal laccase from *Rhizoctonia praticola* between substituted anilines, which are breakdown products of many xenobiotics, and carboxyphenols, which represent humus constituents. Previously, we have shown cross-coupling between 2,4-dichlorophenol and various halogenated anilines (*12*). It was shown that 2,4-dichlorophenol was first oxidized by the fungal enzyme to various halogenated quinones (*19*), which reacted chemically with different anilines. A similar assumption was made in the present investigation. The cross-coupling products detected constituted dimers between a quinone from a carboxyphenol and an aniline (Figure 2). Subsequently, the dimer could be reduced or further coupling reactions could take place.

Depending on the enzyme concentration and the length of incubations, the hybrids produced varied. Our observations with use of more enzyme units and longer incubation times always resulted in the formation of high molecular weight oligomers or polymers, which made the identification of products difficult if not impossible. In this study, the hybrids obtained were the solvent-extractable compounds, and we did not attempt to analyze the water-soluble hybrids.

There were inherent differences in the reactivities of the carboxyphenols, which affected the cross-coupling reactions between them and anilines. It was not surprising that

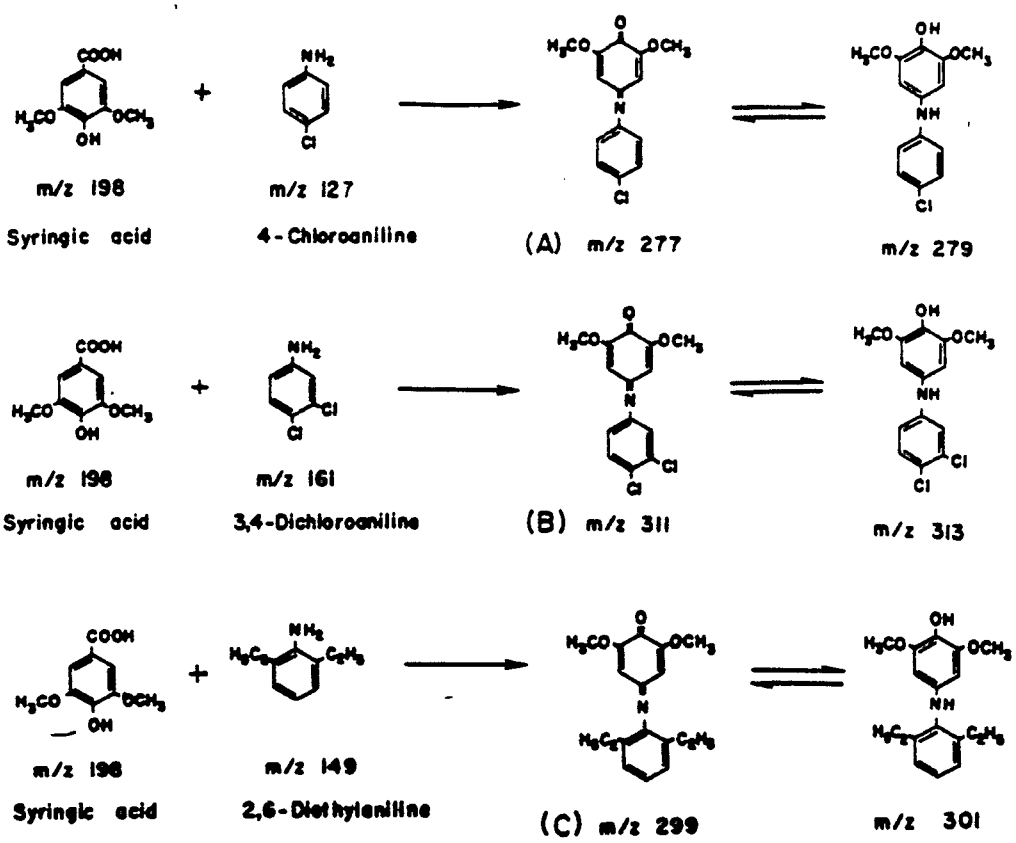


Figure 2. Suggested structures of the hybrid products between syringic acid and variously substituted anilines.

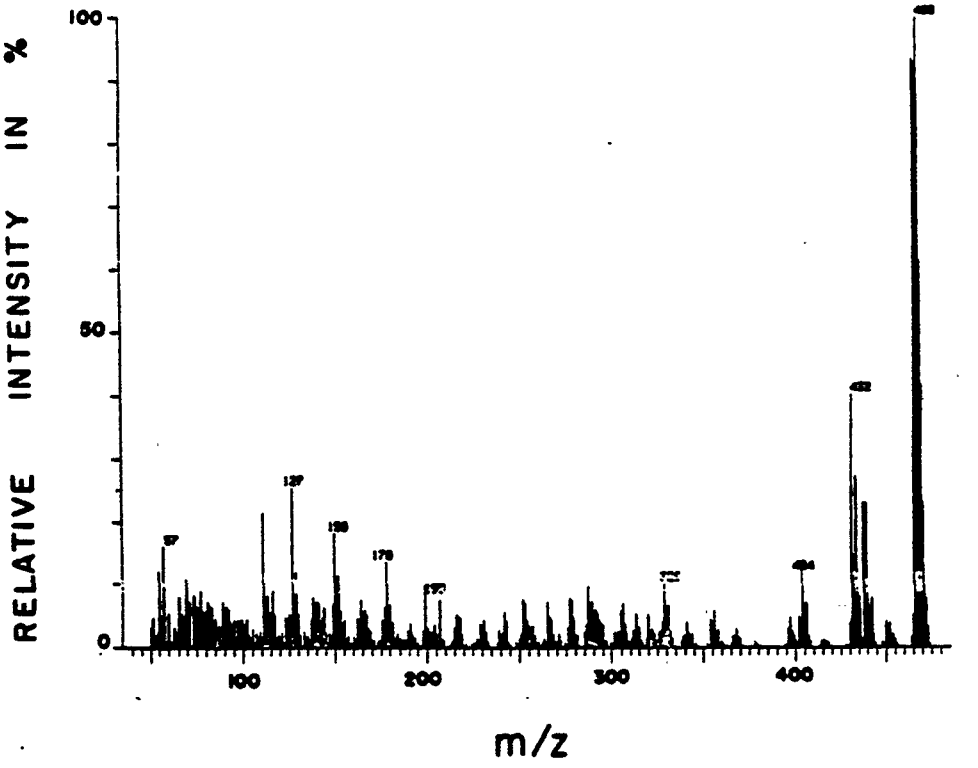
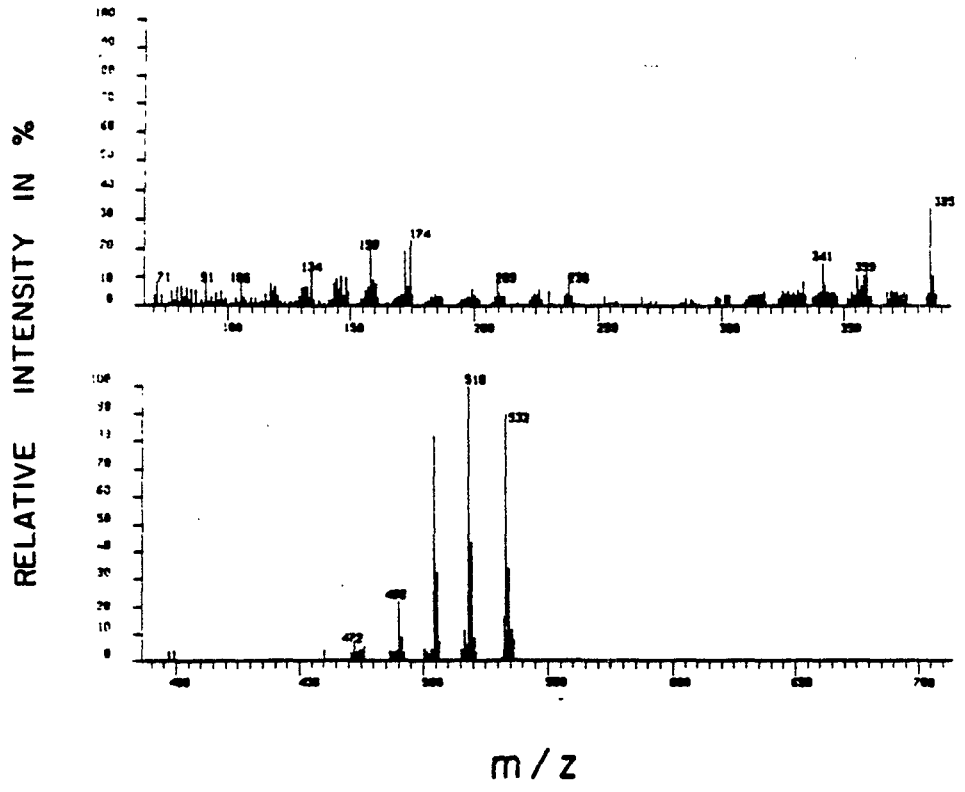


Figure 3. Mass spectrum of a tetramer resulting from cross-coupling between 4-chloroaniline and protocatechuic acid.

vanillic acid formed various hybrid products with anilines because a variety of oligomers were formed when vanillic acid alone was reacted with the laccase (*14*). However, it was rather unusual that relatively few hybrid products were obtained when syringic acid was coupled with the various anilines, considering the diversity of oligomers that could be isolated from syringic acid–enzyme reactions (*15*). This phenomenon was very obvious when syringic acid was coupled with 2,6-diethylaniline. In this reaction, a quin dimer (m/z 299) was produced and accumulated. T compound was resistant to further oligomerization, and this is very likely due to the steric hindrance of further nuclear attack by the ethyl groups and the additional methoxy group. The yield of hybrid dimer (m/z 299) was affected by the enzyme and substrate concentrations (Figure 5, Table VII). The highest yield of hybrid dimer



gure 4. Mass spectrum of a tetramer resulting from cross-coupling between 2,6-diethylaniline and protocatechuic acid.

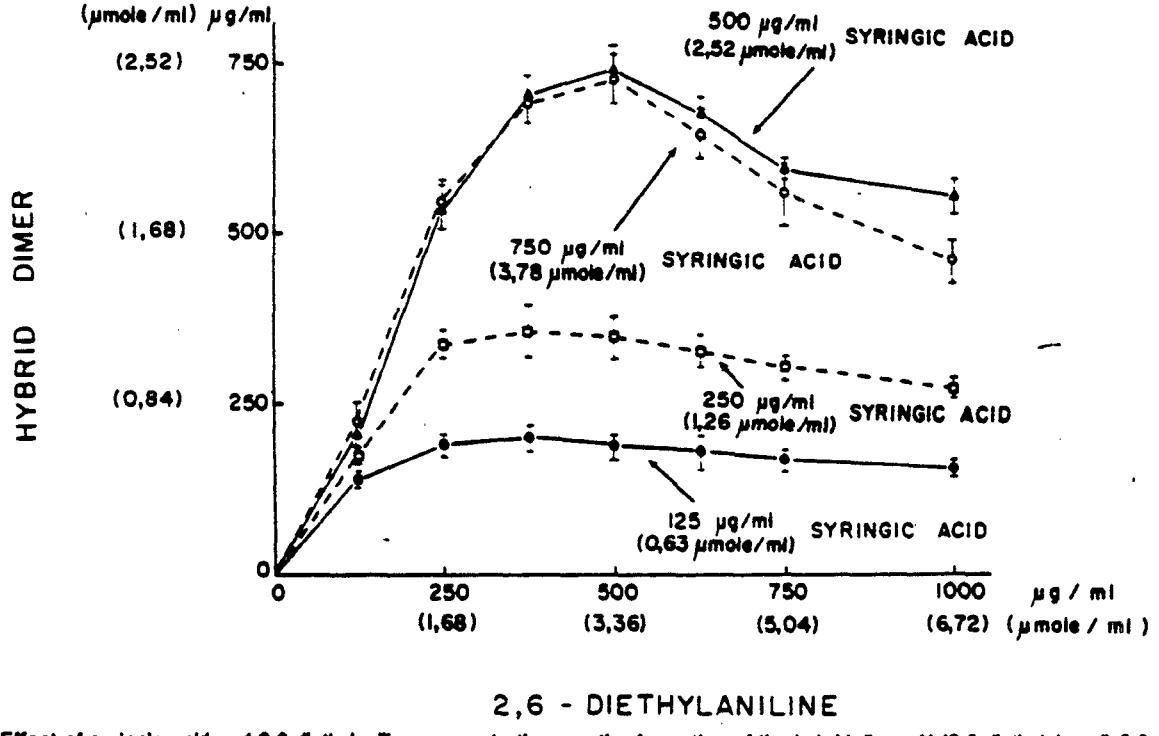


Figure 5. Effect of syringic acid and 2,6-diethylaniline concentrations on the formation of the hybrid dimer *N*-(2,6-diethylphenyl)-2,6-dimethoxy-*p*-benzoquinone imine (*m/z* 299). Bars indicate standard error of the mean of three replicates.

at specific enzyme and substrate concentrations was approximately 86%.

e hybrids identified show that the binding of an an- to a quinone often occurs via bond formation between the nitrogen atom and an aromatic ring carbon resulting in the formation of an imine (anil). This observation has been made previously during the transformation of anilines originating from pesticides. Briggs and Ogilvie (*20*) reported that 3-chloro-4-methoxyaniline, the parent amine of the herbicide metoxuran (*N'*-(3-chloro-4-methoxyphenyl)-*N,N*-dimethylurea), was oxidized in a soil slurry or in a peroxidase assay to 3-chloro-*p*-benzoquinone 4-(3-chloro-4-methoxyanil). Iwan et al. (*13*) extracted two monoimine-type compounds as well as an azo derivative from soil, which was incubated with 4-chloro-o-toluidine. The formation of an anil was also suggested by Hsu and Bartha (*7*) and Parris (*8*) in their studies on the binding of anilines to humic compounds.

While Briggs and Ogilvie (*20*) and Iwan et al. (*13*) found the imine generated in soil samples to be a result of the

coupling of two aniline molecules, our investigation provided evidence for cross-coupling of a quinone derived from a humus constituent and an aniline. It appears that the likelihood of extracting from the soil an imine-type compound composed of a soil phenolic constituent and an aniline is remote, since such a cross-coupling product would be quickly incorporated into the matrix of organic matter. This would explain the relatively fast and strong binding of anilines in the soil. The results from these in vitro experiments serve as a model for demonstrating and explaining an important process occurring in the soil environment.

Acknowledgments

We thank Alan Freyer for the NMR analyses and Ichen G. Chang for help with the HPLC.

Registry No. A, 83560-93-4; B, 83560-94-5; C, 83560-95-6; D, 83560-96-7; E, 83560-97-8; CA, 106-47-8; DCA, 95-76-1; DEA, 579-66-8; V, 121-34-6; Fer, 1135-24-6; Sy, 530-57-4; Prot, 99-50-3; Gal, 149-91-7; laccase, 80498-15-3; α-resorcylic acid, 99-10-5; β-resorcylic acid, 89-86-1; caffeic acid, 331-39-5; phloroglucinol, 108-73-6; resorcinol, 108-46-3; orcinol, 504-15-4.

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Received for review May 3, 1982. Accepted September 7, 1982. Primary funding for this research project was provided by the Office of Research and Development, Environmental Protection Agency (EPA; Grant No. R-808165). The EPA does not necessarily endorse any commercial products used in the study, and the conclusions represent the views of the authors and do not necessarily represent the opinions, policies, or recommendations of the EPA. Additional funding was provided by the Biological Instrumentation Program of the National Science Foundation (Grant No. PCM-8018704) and by the Pennsylvania Agricultural Experiment Station (Journal Series No. 6425).

Dihaloacetonitriles in Drinking Water: Algae and Fulvic Acid as Precursors

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■ Ten chlorinated drinking water samples from diverse locations in southern Ontario have been found to contain dihaloacetonitriles, DHAN's. All samples contained $CHCl_2CN$ (range 0.3–8.1 ppb) and some samples contained CHBrClCN (range not detected to 1.8 ppb). The average molar DHAN concentration was about 10% of the average molar trihalomethane, THM, concentration. Aqueous chlorination experiments conducted on aquatic fulvic acid and two species of aquatic algae (a blue-green and a green) showed that these materials yielded DHAN's under conditions similar to those used for water treatment.

Introduction

The presence of dihaloacetonitriles, DHAN's, in chlorinated water supplies was reported recently by Trehy and Bieber (*1–3*). The apparent reason that these compounds had not previously been found is because they decompose on commonly used gas chromatographic phases such as OV 101 and hydrolyze at elevated temperatures and pHs (*1*). Trehy and Bieber (*2*) showed that dichloroacetonitrile, $CHCl_2CN$, was produced in good yield from the aqueous solution chlorination of amino acids such as aspartic acid and have proposed a reaction mechanism. When bromide was added to the amino acid/chlorine solution at the start of the reaction, a mixture of dichloroacetonitrile ($CHCl_2CN$), bromochloroacetonitrile (CHBrClCN), and dibromoacetonitrile ($CHBr_2CN$) was produced. $CHCl_2CN$ has been shown to be mutagenic in bacterial assays (*4*), so the presence of these compounds in drinking waters would appear to be undesirable.

To date, most investigators of chlorination byproducts have focused their attention on the trihalomethanes, THM's (*5–9*). This paper reports the concentration of dihaloacetonitriles in several southern Ontario water supplies and shows that these compounds can be produced by chlorinating aquatic humic substances and algae under conditions used for water treatment.

Experimental Section

$CHCl_2CN$ and $CHBr_2CN$ were purchased commerically from ICN Pharmaceuticals Inc. CHBrClCN was synthesized by reacting cyanoacetic acid with an equimolar mixture of *N*-chlorosuccinimide and *N*-bromosuccinimide (proportions and reactions conditions of Wilt (*10*)). This reaction yielded a mixture of three DHAN's, which could

APPENDIX B

QUALITY ASSURANCE PROJECT PLAN

QUALITY ASSURANCE PROJECT PLAN

SUPPORT FOR DRAKE CHEMICAL NPL SITE
WORK ASSIGNMENT 22410183

ENVIRONMENTAL PROGRAMS
LOCKHEED ENVIRONMENTAL SYSTEMS & TECHNOLOGIES COMPANY
980 KELLY JOHNSON DRIVE
LAS VEGAS, NEVADA 89119

Contract Number 68-CO-0049

Prepared For

U.S. ENVIRONMENTAL PROTECTION AGENCY
ENVIRONMENTAL MONITORING SYSTEMS LABORATORY
OFFICE OF RESEARCH AND DEVELOPMENT
LAS VEGAS, NEVADA 89119

APPROVAL OF QUALITY ASSURANCE PROJECT PLAN

Environmental Monitoring Research and Development Program
Lockheed Environmental Systems and Technologies

Date: Dec., 1993 QA ID No. 90-93-02-0 Work Assignment 22410183

J.R. Donnelly Task Lead	J R Donnelly Signature	12/13/93 Date
D.R. Youngman QA Coordinator	D. R. Youngman Signature	12/20/93 Date
D.C. Hillman Department Manager	D C Hillman Signature	12/13/93 Date
K. W. Brown EPA-WAM	Kenneth W. Brown Signature	12/21/93 Date
L.R. Williams EPA-QA Officer	_____ Signature	_____ Date

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CONTENTS

1.0 INTRODUCTION

Region 3 has submitted to the EMSL-LV a request for analytical support in determination of ß-naphthylamine (2-aminonaphthalene) in soil, sludge, and thermally treated soil from the Drake Chemical NPL site. At this site, incineration will be performed on approximately 200,000 cubic yards of soil and sludge. The detection level of 1 ppb is desired for quality assurance purposes, and of 55 ppb for routine verification of cleanup. Additionally, p-nitroaniline is sought. This project represents an analytical challenge because standardized methodology has not been developed or validated.

Four objectives have been identified for the support tasked to Lockheed in a memorandum from the WAM dated 9/27/93:

(1) develop an analytical procedure(s) to assure EPA that the incinerator contractor can meet the 55 ppb cleanup criterion on a daily basis, in the field, with the incinerator they propose to use. An ultimate detection limit of 1 ppb would be useful.

(2) analyze samples taken from a location on the site which was not sampled in the previous study (1987).

(3) verify the presence of β-naphthylamine at the site above the cleanup goal level, and the presence at very high levels in certain lagoons.

(4) identify whether nitroanilines are present, such as the <u>para</u> isomer which was found in 1987.

To address the above objectives, Lockheed will research available analytical techniques and methods to identify and test a candidate analytical procedure which will obtain a suitable detection limit for ß-naphthylamine and the nitroanilines. Lockheed will provide an interim report that identifies the approach(es) used to achieve the analytical requirements, including the precise analytical method(s) used, the extraction procedures, and method performance data (detection limits, precision and accuracy).

After a method has been selected it is expected that it will be used to determine the concentrations of the target analytes in approximately 50 environmental samples derived from the Drake NPL site.

1.1 Overview

The primary target analyte, 2-aminonaphthalene, is a white to reddish crystalline solid, mp. 111-113°C, which is volatile in steam and has bp. 294 or 306°C. It is a fluorescent and basic compound with a pK_a of 4.07. It is soluble in hot water, alcohol, and ether, and has a density of 1.0614. Para-nitroaniline is a yellow powder of mp 146°C, soluble in water, alcohol, ether, benzene and methanol. It is used commercially as a dyestuff intermediate.

Under neutral conditions the target analytes should readily extract into a low-polarity or moderate polarity organic solvent such as diethyl ether, hexane, acetonitrile, or methylene chloride; they should also be extractable into water at pH < 2.0. It is expected that the extract could be analyzed by high-performance liquid chromatography (HPLC) or gas chromatography (GC) coupled with mass spectrometry (MS) or other detectors, including UV-vis fluorescence (for 2-aminonaphthalene, using HPLC separation). Fluorescence potentially offers sensitivity and some limited selectivity, with rapid, inexpensive analysis.

Selected-ion monitoring MS (SIM-MS) and high-resolution SIM-MS (HRMS) provide good sensitivity for GC-HRMS determination of both target analytes. This detection method is significantly more sensitive than the quadrupole mass spectrometric detector commonly used in the CLP program.

Method selection will be based upon the Region's requirements for sensitivity and selectivity for the target analytes, combined with the goal of simplicity and ease of performance, in the matrices received from the Drake Site.

1.2 Experimental Design

The experimental design has been developed to evaluate two general areas: (1) sample extraction, and (2) sample analysis. Samples representative of the Drake site will be used to evaluate the selected method.

A factor which may limit or preclude the use of a detector having limited specificity, such as fluorescence, is the possibility of background soil matrix contamination with 2-aminonaphthalene or other fluorescent compounds which interfere with detection of the target analyte. It is expected that the nitroanilines will be less sensitive to fluorescence detection;

this condition could limit the utility of methodology using such a detector. Background soil supplied by the Region will be used as a "blank" matrix so that the method will be developed for the soil type found at the Site.

The extraction method tests will be performed using a mass spectrometric detection system to maximize the information obtained about detectability and interferences. Fluorescence detection will also be used. An isotopic diluent, d_7-β-naphthylamine, will be used for more accurate quantitations and to allow a sample-specific measurement of recovery. Partial separation (ca. 50% valley) of deuterated β-naphthylamine from the native standard has been achieved, so use of the isotopic diluent for mass spectrometric analysis does not render the extract unusable for fluorescence detection. Method testing will be performed until satisfactory methodology is developed. At that point, method performance data will be submitted to the WAM, and approval of that method will be requested.

1.2.1 Sample Extraction Strategy

Two primary sonication-based sample extraction methods will be evaluated: an aqueous acid extraction at pH 1.5 and a neutral SW-846 Method 3550 extraction with an organic solvent found to perform well for the target analytes, such as methylene chloride. Acidic sample extracts will be neutralized and back-extracted into an appropriate organic solvent. Analytical results, based on experiments with standards, will be used to determine which methodology is better.

Subsequent to extraction, sample aliquots will be subjected to gel-permeation chromatography as described in SW-846 Method 3640 or a modified version of SW-486 Method 3610 (an alumina cleanup procedure) if the presence of interferences indicates such cleanup is necessary. This modified procedure will adsorb the amine-based target analytes on acidic silica-gel, and the purified bases will be back-extracted using an acidified methanol/water solvent.

1.2.2 Analytical Procedures

The analytical methods investigation will be based on a series of methods. These are presented in order of the simplest, but least selective, methods first. This order also presents the methods in decreasing order of potential for field deployment. Initially, each technique will be tested briefly for probability

of success. Additional investigations will be based upon the success of early work, to minimize the time and costs required to develop a satisfactory method.

1.2.2.1 Fluorometric Determination

If analysis of soils from the Drake site indicate the absence of potential interferences, fluorescence spectrometry may be investigated in two modes applied directly to the various standards and sample extracts. These investigations will depend upon time and funding availability. The first mode is the traditional dispersive emission spectrometric mode. The approximate excitation wavelength will be 366 nm (the absorption maximum for 2-aminonaphthalene in benzene) and the emission signal at approximately 420 nm (the reference emission maximum under near-neutral conditions ($1 < pH < 10$) (1)) is measured as a function of concentration. The second method of fluorometric determination, synchronous fluorescence, is to scan simultaneously both emission and excitation monochromators at a fixed wavelength separation (to be optimized experimentally for sensitivity). This procedure will result in a signal peak whose area will then be correlated with the concentration.

1.2.2.2 HPLC - Fluorometric or Diode Array Detection

The standards and extracts will first be separated into the various components on reverse-phase HPLC and the fluorescent analytes will be detected in a direct fluorescence monitoring mode with the excitation and emission wavelengths chosen to provide good sensitivity and selectivity, by examining the results of scanning across a wavelength range. This system will be optimized for β-naphthylamine and for the nitroaniline secondary analytes. Mass spectrometric detection is generally applicable and provides mass data for confirmation of identity, which may be more specific than the fluorescence data. Mass spectrometry will be utilized for at least that percentage of the samples which will be subjected to confirmation analysis. Diode array detection will also be tested in conjunction with HPLC separation.

1.2.2.3 GC/(HR)MS

The standards and extracts will first be separated into the various components on capillary column GC, and the analytes will be detected with scan or SIM-(HR)MS. This technology will be used for (a) accurate mass confirmation, if deemed appropriate, and (b) routine analysis, if LC/MS is not available or found unsuitable to meet detection limit or separation requirements.

1.2.2.4 HPLC/MS

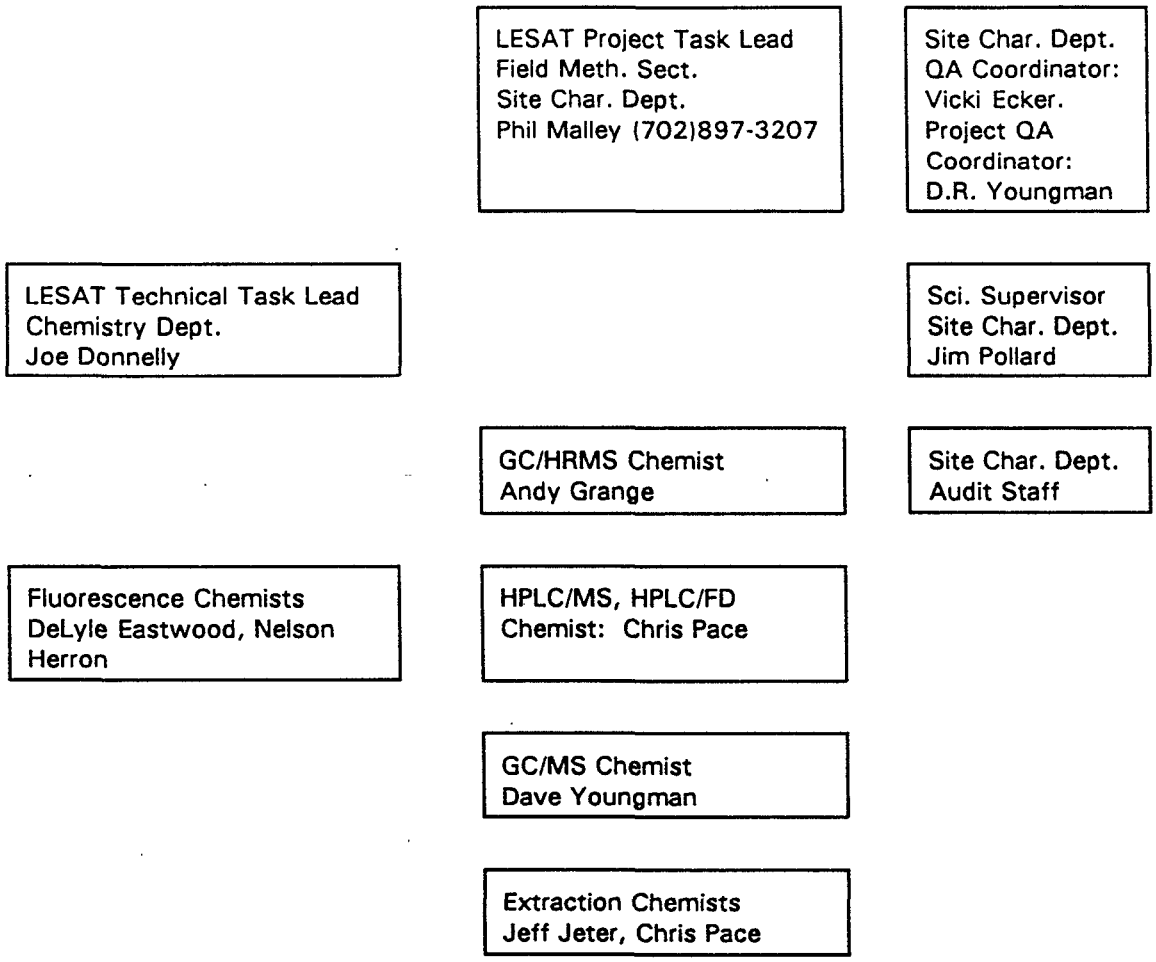
The standards and extracts will first be separated into the various components on reversed-phase HPLC (using the same method as in Section 1.2.2.2, above), and the analytes will be detected with thermospray-quadrupole MS. This technology has been found to provide suitable detection technology for the target analytes: (a) the detector is selective and sensitive (especially in SIM mode); (b) the target analytes give good responses; (c) the separation technique may be powerful enough to minimize time-intensive sample extract cleanup requirements; (d) the readily-oxidized amines are expected to be less subject to degradation with LC than with GC separation.

1.3 Schedule

The LESAT task lead will co-ordinate LESAT activities and manage the over-all project. The LESAT technical task lead will direct technical staff and operations to ensure that daily operations are technically sound, complete, and in compliance with the QAPP. The technical task lead will provide the task lead with a brief technical update weekly. An evaluation of the results of the preliminary studies will be submitted to the WAM by November 30, 1993.

1.4 Project Management

The EPA work Assignment Manager for this task is Mr. Ken Brown. The LESAT project management structure is as follows:



2.0 QUALITY ASSURANCE OBJECTIVES

QA objectives for this research project will be finalized after method performance on background soil is determined. Interim goals can be set with reference to existing EPA methods, however. These goals and objectives will be revised as necessary, based upon performance of the method that is developed, on Site samples provided by Region 3.

Isotope dilution LC/MS, using d_7-β-naphthylamine, would be expected to give data quality similar to that of other isotope dilution methods, such as the EPA uses for dioxin analysis. Recoveries of 25-125%, and a detection limit for quadrupole-SIM-MS on the order of 1 ppb are expected. Duplicate sample results should agree within 50%, and blanks should be free from

contamination at the detection limit. It is anticipated that one blank and one duplicate per sample batch will be analyzed.

Background soil material will be provided by Region 3 and utilized for method development. The matrix will be homogenized by stirring and removing obvious inhomogeneous, foreign objects such as rocks, leaves, or sticks. After initial analysis, sample preparation will be developed to remove analytical interferences found in the background soil. This soil will be used for spiking to demonstrate method performance (recovery, detection limit, reproducibility). The background and field samples will be homogenized in the container as received, if practical. Large rocks, sticks, etc. will be removed and the sample will be stirred with a stainless steel spoon or spatula. This procedure is consistent with other methods, and reasonable for the number of samples (ca. 50) and time/cost constraints of the project.

Results on the background soil will be used to assess whether the interim quality assurance goals can be met within time and cost constraints of the project. Adjustments to these goals will be made if needed, upon approval by the WAM. Upon request, data regarding precision, detection limit, quantification levels reported, and number of replicates analyzed will be used to calculate confidence intervals for reported data.

Interim Method Requirements (Acceptance Criteria)	
Required method detection limit	55 ppb
Ideal method detection limit	1 ppb
Duplicate sample results	± 50%
Blank samples	analytes ND at required MDL
Recovery of spiked analyte	25%-125%
Extract cleanup quality	quantitation unaffected within required duplicate precision level (± 50%)

3.0 SITE SELECTION AND SAMPLING PROCEDURES

No sampling activities will be performed by Lockheed staff under this task. Background/blank samples to be used during method evaluation will be provided by the EPA. The field samples

will not require chain-of-custody procedures, according to Region 3 (Roy Schrock). He stated that the Region may send the samples under Chain-of-Custody simply to have a record that they were shipped to Las Vegas, however.

4.0 ANALYTICAL PROCEDURES AND CALIBRATION

This section discusses the preparation of test samples and extractions and cleanups of those synthetic or surrogate samples. The preparation of these synthetic method-development samples will be performed by Lockheed.

4.1 Site-Specific Background Soil Matrix

As noted in Section 2.0, background soil will be used for method development/testing, so that a suitable method can be identified with minimum cost/time expended, for the Drake Chemical NPL Site matrix.

4.2 Sample Preparation

Analytical samples will be prepared by spiking the sample aliquot with the isotopic diluent (d_7-β-naphthylamine) at a level which will permit accurate isotope dilution quantifications to be performed (3-10 times the method detection limit), and extracting the sample as discussed in Sections 1.2.1 and 4.3.

4.3 Sample Extraction

The following extraction procedures will be tested to arrive at a procedure that performs suitably for the target analytes. Ideally, the extraction will recover the target analytes at levels over 50% and minimize the carry-over of potential analytical interferences into the resultant extract.

4.3.1 Sonication Extraction

SW-846 Method 3550 sonication extraction will be used with an appropriate organic solvent (e.g., methylene chloride), and a 10 to 40-g sample size to achieve adequate detection limits. If this method does not provide adequate recovery or reproducibility, Soxhlet extraction (Method 3540) will be tested.

4.3.2 Cleanup of Method 3550 Extracts

Method 3550 sample extracts will be cleaned up by 3650 acid/base partitioning, as required. If no interferences are observed, no further cleanup will be performed.

Further cleanup steps which will be tested as necessary include SW-846 Method 3640 using gel-permeation chromatography, adsorption onto acidic alumina, back-extraction into methanol/water or acetonitrile/water at pH ≈ 1.5, with neutralization and solvent extraction.

4.3.3 Sonication Extraction into Aqueous Acid

The spiked sample will be ultrasonically extracted with pH 1.5 hydrochloric acid as per instructions in SW-846 Method 3550, Sections 7.4.2 - 7.4.5. The combined extract will then be brought to a pH of 10 by the addition of aqueous NaOH.

4.3.4 Cleanup of Acid Extracted Solutions

The pH 10 extract will be immediately subjected the procedure specified in Method 3650 but the organic bases will be extracted into an introduced organic solvent.

If significant interference remains, the extracts will receive further cleanup by SW-846 Method 3640 (gel-permeation chromatography) or the acid-alumina method (Section 4.3.2).

4.4 Analytical Measurements

Specific calibration QA/QC required for instrumental analysis procedures will be consistent with those for standard EPA methods utilizing the technology. For examples, HRMS instrument calibration will be performed with perfluorokerosene (PFK), as for chlorinated dioxin analysis; LC/MS calibration will follow the recommendations elaborated by Lockheed under this EPA contract for high-mass PAH analysis (QAPjP and reports generated under J.O. 7078). Concentration calibrations will be similar to other isotope dilution methods, such as for dioxins, and will employ a three-point calibration curve over the concentration range of interest.

It is anticipated that dispersive fluorescence and synchronous fluorescence will be tested to determine whether this potentially rapid analytical technique is applicable to these samples.

4.4.1 Dispersive Fluorescence Detection

In this method an aliquot of the sample extract is placed in a cuvette in a darkened chamber and irradiated with a selected bandwidth of light. The excitation source beam is wavelength-dispersed by a monochromator grating oriented so that the desired portion of the dispersed beam passes through a slit-like beam stop. This light is chosen so that the band overlaps the most highly absorbing portion of the absorption spectrum of the target molecule. Certain complex, rigid planar molecules with aromatic π-bonding such as 2-aminonaphthalene re-emit the absorbed energy in a process called fluorescence. The emitted light is collected at a 90° angle to the excitation beam by imaging it onto another grating dispersion system that allows a desired wavelength of fluorescence emission to be detected by a photomultiplier tube.

The emitted wavelength is at a somewhat lower energy and longer wavelength than the absorbed photons of light. This is due to some leakage of the absorbed energy into internal molecular motion (non-radiative processes). The advantage of this wavelength shift is that the emitted light is that there is little background scattered light, so very sensitive light detection circuits can be used to detect the fluorescence, providing lower detection and quantitation limits than are generally available through optical absorption techniques. A second advantage is that fewer molecules fluoresce than absorb light. Therefore, fluorescence detection has a relatively higher degree of selectivity than absorption spectrometry. A third advantage of fluorescence detection over many current analytical methods is that it can have a much higher sample throughput (and often less sample preparation) than the chromatographic methods.

However, fluorescence of whole extracts does not exhibit the selectivity associated with chromatography coupled with mass spectrometric detection. Therefore, the method must be evaluated in terms of the site-specific matrix. To achieve this goal, the fluorescence detection method will be evaluated in terms of blank soils spiked with known amounts of β-naphthylamine. Because fluorescence methods cannot differentiate between isotopically labelled and native forms of a compound, the samples extracted for fluorescence detection cannot be fortified with d_7-β-naphthylamine. However, naphthalene or α-naphthylamine could be used.

The literature contains many references to the fluorescence of 2-aminonaphthalene(1). The absorption maximum for the excitation beam appears to be approximately 366 nm in a non-polar organic solvent and the emission maximum appears near 420 nm for

pH values between 1 and 10. The excitation and emission wavelengths will be optimized for the target analyte in the selected solvent, and the slit widths will be optimized to maximize fluorescence throughput while minimizing the background stray light.

The fluorescence instrument, a Perkin-Elmer LS-50, will be calibrated each day prior to any analyses. A 40-g sample of soil contaminated at 55 ppb will yield 2.2 mg/L of 2-aminonaphthalene if recovery is 100%. This concentration of analyte should be easily determinable by fluorometric assay. The anticipated calibration method for the project is a minimum of four concentrations of 2-aminonaphthalene in cyclohexane ranging from 0.5 mg/L, the lower quantitation limit at approximately 20 percent recovery to 6.5 mg/L, the upper quantitation target at 100 percent recovery. This range may need to be adjusted with further experience. The existing QAPjP for the calibration of fluorescence instrumentation will be used.

4.4.2 Synchronous-Scanning Fluorescence Detection

The wavelength separation between maximum absorbance and maximum fluorescence is a an increasingly selective indicator for a given compound. These two properties are combined in synchronous scanning fluorometry to provide increased selectivity in fluorescence analysis. The target analyte may be quantitated by peak area or peak height, depending on the "purity" of the spectral peak. The precise quantitation method (area or peak height) will be made after examining fluorescence spectra of spiked soils from the site.

The instrument, wavelength and bandpass selections, and the calibration regimen will be the same as that covered in Section 4.4.1.

4.4.3 HPLC with Fluorescence, UV-Vis, or Diode Array Detection

HPLC separation of the target analytes from interfering chemicals may be needed for a fluorescence detection method. Multiple wavelength separations on multiple elution windows may be used for more complicated systems such as the simultaneous determination of nitroanilines (a secondary project goal). Systems that use integrated circuit detectors perform this function mathematically. The evaluation of HPLC/FD will be performed using a Hewlett-Packard system equipped with a standard single-channel photodetector. UV-vis and diode array detection will also be tested, as straightforward detection systems for

HPLC which are potentially less time- or cost-intensive than mass spectrometric detection.

HPLC solvent systems are generally incompatible with non-polar sample solvents. Therefore, when samples are extracted for HPLC analysis, the final 1 mL extract of analyte/organic solvent solution will be extracted with a suitable acidified solvent system such as such as three times 0.5 mL of methanol/water or acetonitrile/water at a pH ≈ 1.5 (see Sections 4.3.2 and 4.3.4 for similar procedures).

4.4.4 HPLC with MS Detection

Typical GC/MS methods are limited in their soil PQLs due to the limited injection volume used (1 - 2 μL). HPLC has the advantage that relatively large samples can be loaded into a sampling loop for mechanical injection without the serious degradation of chromatography observed in GC. A conservative 50 μL maximum injection from a 40-g sample of soil that was 100 percent extracted and passed through the cleanup procedure would deliver 110 ng of 2-aminonaphthalene, which is typically very easy to measure with a quadrupole mass spectrometer. An extraction efficiency of 20 percent will still deliver 22 ng of analyte, which is greater than that delivered in the current lowest CLP standard (20 ng of analyte).

The HPLC/MS studies will be performed using a Hewlett-Packard system consisting of a 1090L liquid chromatograph, Thermospray Interface, and 5988A mass spectrometer. The system will be calibrated using an initial calibration (I.C.) program containing a minimum of three standards. The lowest level standard should deliver no more than 20 ng of analyte and the high level standard should deliver 200 to 300 ng of 2-aminonaphthalene if this amount is in the linear range of the instrument. If this upper level is outside linear range, then the high-level standard should be at the upper limit of the linear range. An internal standard such as 1-aminonaphthalene or acenaphthene-d_{10} will be added so that each injection contains a fixed amount of this material (preferably 100 ng). A continuing calibration (C.C.) procedure is performed each day analyses are performed after the I.C. has been performed. The C.C. standard should give a response approximately equal to that of the analytical extract from the 55 ppb soil. In all standards the relative response factor (RRF) will be calculated as:

$$RRF = (R_A * M_{IS}) / (R_{IS} * M_A) \quad (1)$$

where

R_A, R_{IS} Response of analyte and internal standard, respectively

M_A, M_{IS} Mass of analyte and internal standard, respectively

The acceptance criteria for the I.C. is that the relative percent standard deviation (%RSD) of the RRFs from the process not exceed 30%. The acceptance criterion for a valid C.C. is that the percent difference (%D) between the RRF for the C. C. and the average RRF for the I.C. cannot exceed 25%. These acceptance criteria are derived from the criteria for mass spectrometer calibration in SW-846 Method 8270 for semivolatiles.

If the thermospray interface does not appear sufficiently stable to meet these criteria, alternate criteria specified in EPA-600/X92/102 will be followed. This procedure specifies that each standard will be injected three times and the average RRF for each standard will be specified for the calibration curves.

I.S. areas in any C.C. standard, sample, or blank (also called samples for QC evaluations) should not vary by more than -50% to +100% from the average of the internal standard responses for the I.C. If this occurs the sample will be reanalyzed twice. If the average I.S. response is still outside this criterion, the system will be repaired, a new I.C. will be performed, and analyses will be resumed after the last acceptable sample analysis.

All sample extracts will be extracted into an acidified organic solvent/water system as described in Section 4.4.3.

4.4.5 GC with (HR)MS Detection

The primary target analyte, 2-aminonaphthalene, is contained on the target analyte list for SW-846 Method 8270, but the practical quantitation limit (PQL) is not estimated. However, the general PQL for these analytes in soils using scanning quadrupole mass spectrometry is in the range of 700 ppb, according to the Method 8270, Table 2, when using a mass-scanning mode from 35 to 500 daltons. For greater sensitivity, two gas chromatography/mass spectrometry systems may be investigated in SIM mode. One will be a standard Hewlett-Packard capillary-column GC (DB-5 or equivalent) with a quadrupole mass detector and the other will be a VG 70-250SE system with a capillary column (DB-5 or equivalent) and a high-resolution, double-sector mass spectrometer. It is anticipated that the internal standard

acenaphthene-d_{10} as recommended in SW-846 Method 8270 will be suitable.

The %RSD acceptance criteria for the I.C. will be 20. This criterion is a compromise between the criteria specified by the general Method 8270 (30%) and that specified in the SIM procedure in Method 8280 (15%). A more liberal %D criterion of 30% must be met for a C.C. to be accepted. This criterion is adopted directly from Method 8280.

I.S. areas in any C.C. standard or sample (including blanks) should not vary by more than -50% to +100% from the average of the internal standard responses for the I.C. If this occurs the sample will be reanalyzed. If the I.S. response is still outside this criterion, the system will be repaired, a new I.C. will be performed, and analyses will be resumed after the last acceptable sample analysis.

5.0 DATA VALIDATION, REDUCTION, AND REPORTING

The data derived from the initial phase of this project will be used to determine validation criteria for the field samples from the Drake Site. The data will be collected by senior staff and they will be reviewed by the task technical lead and the project QA lead. The data will be reduced by a variety of methods ranging from comparing optical emission intensities at selected wavelengths with standard data to mass spectral data reduction algorithms. However, all reports containing reduced data will reference standard EPA calculation techniques or provide demonstration calculations for each step of the data reduction.

All reduced data contributing to the final evaluation will be reported on an as-received (wet) soil weight basis in µg/kg (parts-per-billion, ppb) units.

The final report will provide tabulated data for each sample and for the associated quality assurance measurements.

6.0 INTERNAL QUALITY CONTROL CHECKS

Research projects, such as this one, rely on the judgments of senior staff to provide ongoing assessments of experimental results so that successful method development is achieved in a minimum amount of time, and with lowest cost. The results of these assessments, and the strategies used, will be presented in the project reports. Technical cross-talk with the WAM and EPA

technical advisors selected by the WAM will be used as well, so that by discussions and agreements, the best technical approaches will be investigated, optimized, and presented.

6.1 Blanks

This project will utilize instrument and method blanks to ensure that the data are valid and usable. Blanks must be clean to the method detection limit, or quantification limit to be used for the associated data. At least one method blank will be analyzed with each batch of samples. A batch is defined as a group of samples processed together. The method blank will be spiked with the surrogate or isotopic diluent (for those techniques using the diluent), and in all cases will be analyzed in the same manner as the field samples associated with it.

6.2 Duplicates

At least one duplicate field sample will be analyzed per sample batch. Reproducibility must be ± 50% between the two results. At least three spiked background samples will be used to test methods in the method development phase of the project.

7.0 PERFORMANCE AND SYSTEMS AUDITS

The primary target analyte, 2-aminonaphthalene, and the analytical methods, GC/HRMS, HPLC/MS, and HPLC-FD, are not currently contained in any performance evaluation program. Therefore, no performance audits will be performed on this task.

The LESAT task lead (Section 1.0) will work with his department management to schedule one systems audit of the project. The purpose of this audit will be to ensure that the systems are producing data which are in control relative to this QAPjP. Personnel to conduct the audit, and interpretation of results will be the responsibility of the project QA coordinator and the department manager.

8.0 CALCULATION OF DATA QUALITY INDICATORS

The assessment of data quality with respect to the QA goals stated in Section 2.0 will be conducted using several approaches. The results of different types of sample analyses or measurements will be used to assess completeness, accuracy, precision, and MDLs. The assessment procedures to be used on the results

generated from the measurements and the applicable calculations are described below.

8.1 Completeness

For this project, the degree of completeness will not refer to completeness of site sampling, but only to the completeness of analysis of the samples that are received. At this level, completeness is based on the amount of valid data obtained from those samples or measurements actually collected. Incompleteness is due to samples or measurement data associated with unacceptable QC analyses or a measurement system that is out of statistical control.

Completeness will be calculated by:

$$\%C = 100 \times (V/n) \qquad (2)$$

where:

%C = percent completeness
V = number of measurements judged valid
n = total number of measurements necessary to achieve an acceptable and technical level of confidence.

Specific completeness goals cannot be specified at this time due to present lack of a validated method and the potential variability of sample concentrations (ppb to percent levels are anticipated) and analytical interferences. In general, it is the goal of the investigation to provide usable data for at least 90% of the samples received, within scientific possibilities and time allowed.

8.2 Accuracy

Accuracy will be assessed by evaluating the isotope dilution (spike) results for recovery, relative to the internal standard, and by comparing results on duplicate samples.

8.3 Precision

The precision of each of the analytical methods employed in the analysis of the Drake Site samples will be evaluated by duplicate analysis results.

The relative percent difference may be calculated by:

$$\%D = 100(C_1 - C_2) / (C_1 + C_2)/2$$

where:

%D = relative percent difference
C_1 = larger of the two observed values (or % recoveries)
C_2 = smaller of the two observed values (or % recoveries)

8.4 Detection Limits

Two main types of detection limits will be used in assessing data quality and method performance in this study, Method detection limits (MDLs) and instrument detection limits (IDLs). The MDL is calculated from data obtained on samples from the Drake Site, which contain the analyte at a concentration that produces a signal at three times the instrument noise level. The IDL corresponds to the amount of analyte which would produce a signal three times the noise level when measured by the instrument.

9.0 CORRECTIVE ACTIONS

Corrective actions will consist of any actions taken to correct systematic program problems that are (1) identified by the task technical lead in consultation with the technical staff or (2) identified by the QA oversight staff during an audit function. Data review procedures to determine the deficiency's impact on data integrity shall be identified. Corrective actions shall include reanalysis or different analytical procedures to arrive at usable data.

10.0 QUALITY CONTROL REPORTS TO MANAGEMENT

Because this project does not depend on routine analyses according to a fixed set of QC criteria, standard control chart evaluation procedures and reports to management are not applicable. However, the senior analytical research chemists will report progress and instrument status regularly to the task lead. Whenever the response for the selected parameter falls near or outside the control limit, this will be reported and corrective actions, such as repair and maintenance of the equipment, will be performed.

11.0 REFERENCES

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