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PREFACE

Aquatic fulvic acid, the soluble organic material of natural waters that is not extractable into organic solvents and that remains dissolved on acidification with mineral acid (ref.1), is believed to be a complex mixture of natural products, and to be responsible for the binding and transport of many non-polar organic pollutants (ref.2). My research objective is to better understand the composition of fulvic acid, be able to identify differences and similarities between fulvic acids obtained from different sources, as well as to identify chemical properties believed responsible for the binding of organic pollutants.

During the time spent doing research I found that there were four separate and distinct phases associated with my work . For this reason I have chosen to divide my report into four sections. Section 1 is an introduction as well as a description of preliminary experiments chosen to help determine the direction my research should take. Section 2 presents methods of functional group analyses chosen to characterize fulvic acid. Section 3 is an expanded version of one specific method for micro determination of carboxyl groups in fulvic acid. (Sections 2 and 3 are research papers submitted for publication and are included in my report.) The final section, section 4, is work related to binding studies associated with fulvic acid. I have also presented a summary of my work in hopes of helping the reader to understand the relationship existing between each section.

My research was made possible by an appointment at the National

Institute of Environmental Health Sciences. All work was done in the laboratory of Dr. Phillip Albro, who also served as my research advisor. The fulvic acid used for part of my research was provided by the Humics Group at the University of North Carolina at Chapel Hill, headed by Dr. Russell F. Christman and Dr. J. Donald Johnson who both served in an advisory capacity on my committee. With the assistance of these people as well as help from the aforementioned institutions I was able to successfully accomplish my research program.

SECTION I

1.1 INTRODUCTION

The background information I obtained indicated there is much to be learned concerning methods of functional group characterization for fulvic acid. The nature of such a complex material is not well known and different research groups report different information concerning the characterization of fulvic acid. This is probably due to the various sources from which the fulvic acid is obtained as well as the variety of methods used.

I began my research by purchasing Aldrich Humic Acid (Aldrich Chemical; Milwaukee, Wi.) from which I extracted fulvic acid with a modification of Thurman and Malcolm's separation method (ref.1,3). I did this because of the difficulty of obtaining a natural aquatic fulvic acid (eg. Singletary Lake) at this point in time. Once extracted, some of the more commonly-used types of analyses were performed. Many of these methods turned out to give misleading results and will be discussed briefly in my experimental section. Some of these techniques however proved to be valuable for further research.

I originally intended to functionally identify a large portion of the fulvic acid structure. (Lake Singletary fulvic acid was subsequently obtained and the use of Aldrich material discontinued.) I was also interested in functional groups unique enough to enable one to easily quantify trace quantities of fulvic acid. The usefulness of being able to quantify trace amounts of fulvic acid is apparent in studies using HPLC. For example, often one questions

whether a portion of the fulvic acid remains on an HPLC column after the separation is believed to be complete. If one could quantify fulvic by a simple yet sensitive method determining accurately the ratio of the functional group of interest to the total weight of fulvic acid, the percentage of fulvic acid recovered from an HPLC column could be determined. This would be valuable for determining any small quantity of fulvic acid too small to be accurately weighed. The first section of my report will examine preliminary steps taken towards these types of analyses while sections 2 and 3 expound on actual functional group identification methods used.

1.2 PRELIMINARY EXPERIMENTS

1.2.1 Extraction of Fulvic Acid from Aldrich Humic Acid Sodium Salt

One gram of Aldrich Humic acid sodium salt (Aldrich Chemical) was dissolved in a one liter graduated cylinder filled with de-ionized/distilled water. While stirring constantly HCL was added to bring the pH of the solution to 1 (approximately 50 ml HCL). The cylinder was capped and refrigerated (approximately 4^{o} C) allowing the precipitate to settle overnight. The following day the supernatant fraction was carefully vacuum filtered through Whatman filter paper #1 and the remaining slurry poured into centrifuge tubes and spun at 3000 rpm for 20 minutes in a refrigerated centrifuge at 4^{o}C. The supernatant in these tubes was poured through the filtering apparatus used previously and added to the supernatant previously collected. The precipitate was discarded.

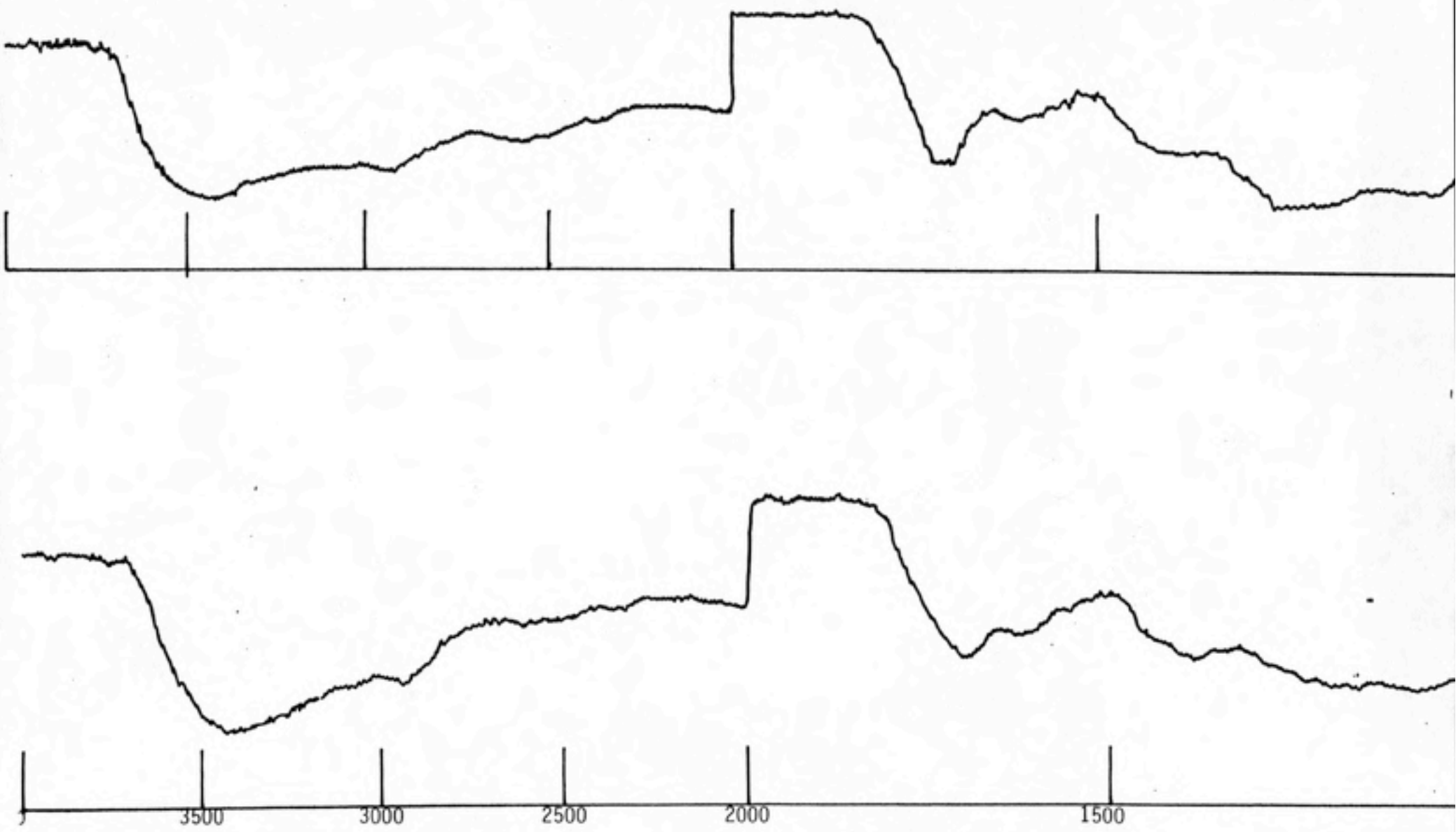
The supernatant was concentrated on a pre-cleaned XAD-8 resin

column and eluted with 0.1N NaOH solution. Five milliliter fractions were collected from the column and fraction collection was discontinued when no more color was detectable in the elution. The brownish-yellow fractions were combined, neutralized with 0.1N HCL and freeze-dried overnight.

The following day the freeze-dried material was dissolved in as little de-ionized/distilled water as possible and put through an ion-retardation column (AG-11, A8, 50-100 mesh, Bio-Rad, Richmond, Ca.), eluted with water collecting 2.5 mL fractions until no more distinguishable color was observed. Starting with the fractions collected last, working backwards, towards the first fractions collected, a few drops of concentrated Silver Nitrate solution (saturated $AgNO_3$, Aldrich Chemical, in de-ionized/distilled water) was added to each tube to detect the presence of free chloride ion by forming a white silver-chloride precipitate. I expected the chloride ion to be detected in the almost colorless fractions with a decreasing concentration detected as the more yellowish colored fractions were tested. At some point prior to testing the yellowish-brown fractions, I predicted the chloride ion would be non-detectable. I discovered however the chloride ion was not adequately separated from the fulvic acid the first time through the column. I therefore combined all the non-tested yellowish-brown fractions, freeze-dried them, and eluted them through the AG-11 column as before. Usually this second elution proved more successful for separation of excess chloride ion from fulvic acid. After a final freeze-drying process the fulvic acid was ready for analysis.

Figure 1 (top). I.R. Spectrum,Aldrich Fulvic Acid

Figure 2 (bottom). I.R. Spectrum, Singletary Lake Fulvic Acid



An infrared (IR) spectrum of the fulvic acid (fig.1) was run by making a KBr (Perkin-Elmer) pellet and using a Perkin Elmer 3,000 infrared spectrophotometer. I later compared this spectrum with a similar spectrum of Singletary Lake Fulvic Acid (fig.2).

Further purification was accomplished by dialysis. A portion of the fulvic acid was dissolved in de-ionized/distilled water and put in a dialysis bag with a molecular weight cut-off of 6,000-8,000 (spectrapore 1, Fisher Scientific). This was allowed to dialyze against water for a period of 72 hours. The fraction remaining inside the bag (dialysis-in) was removed, freeze-dried and an IR spectrum (fig.3) was run in a similar manner to the un-dialyzed fulvic acid The fraction outside the bag (dialysis-out) was also freeze-dried, an IR spectrum also run and compared to the dialysis-in fraction (fig.4).

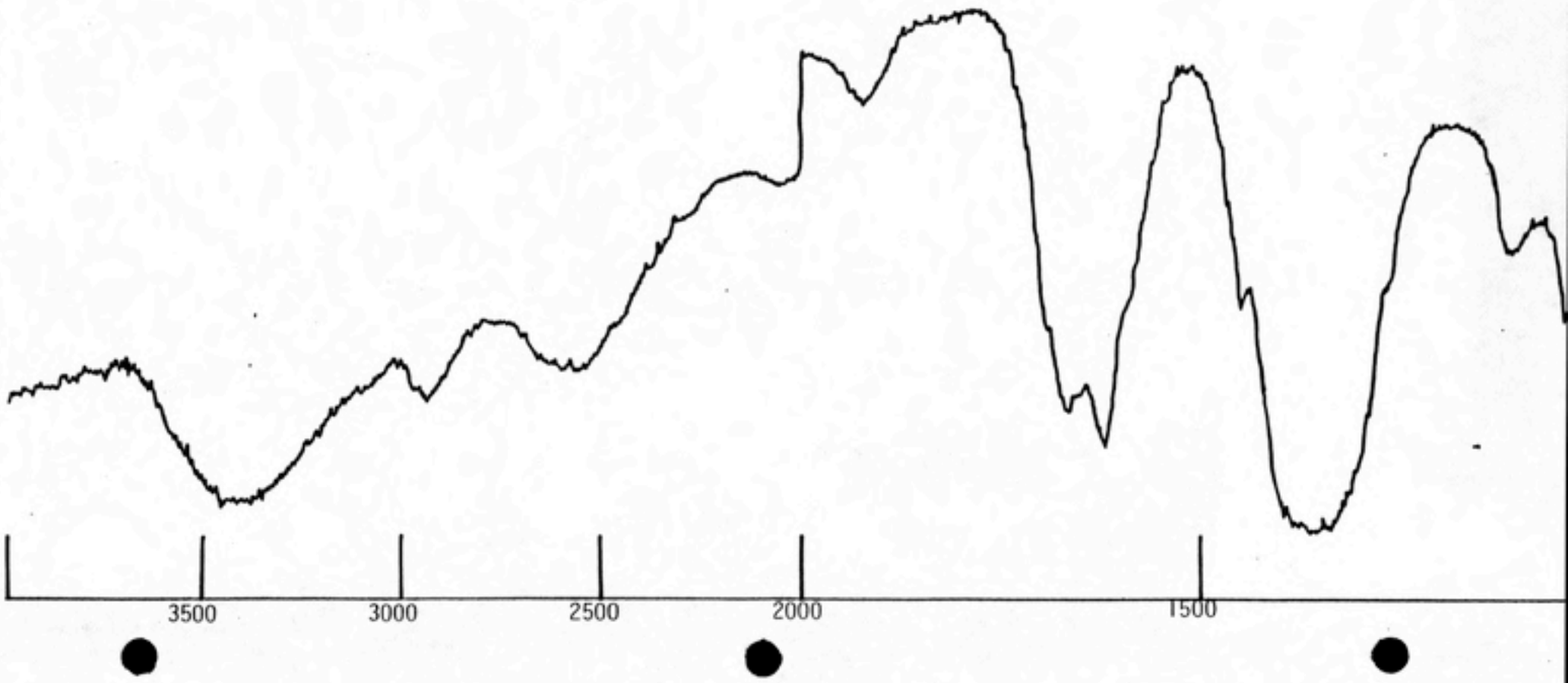
1.2.2 Ash Content

In anticipation of being able to characterize a major percentage of fulvic acid into specific functional groups I concluded it would be desirable to quantify the non-oxidizable portion as well. For each fraction, dialysis-out and non-dialyzed Aldrich fulvic acid and Singletary Lake fulvic acid, I carefully weighed about 10 mg into a dry crucible (previously weighed) and recorded the weight of each sample to the nearest 0.1 mg. I placed the crucible with fulvic acid over a bunsen burner for about 3-5 minutes being sure all oxidizable material had dissipated. The crucible was then placed in a desiccator and weighed when cool. The ash content was computed as follows:

$$\frac{\text{wt. of non-oxidized fulvic acid}}{\text{total wt. of fulvic acid}} \times 100 = \text{Ash Content}$$

Figure 3. I.R. Spectrum, Aldrich Fulvic Acid (dialysis-in)

Figure 4. I.R. Spectrum, Aldrich Fulvic Acid (dialysis-out)



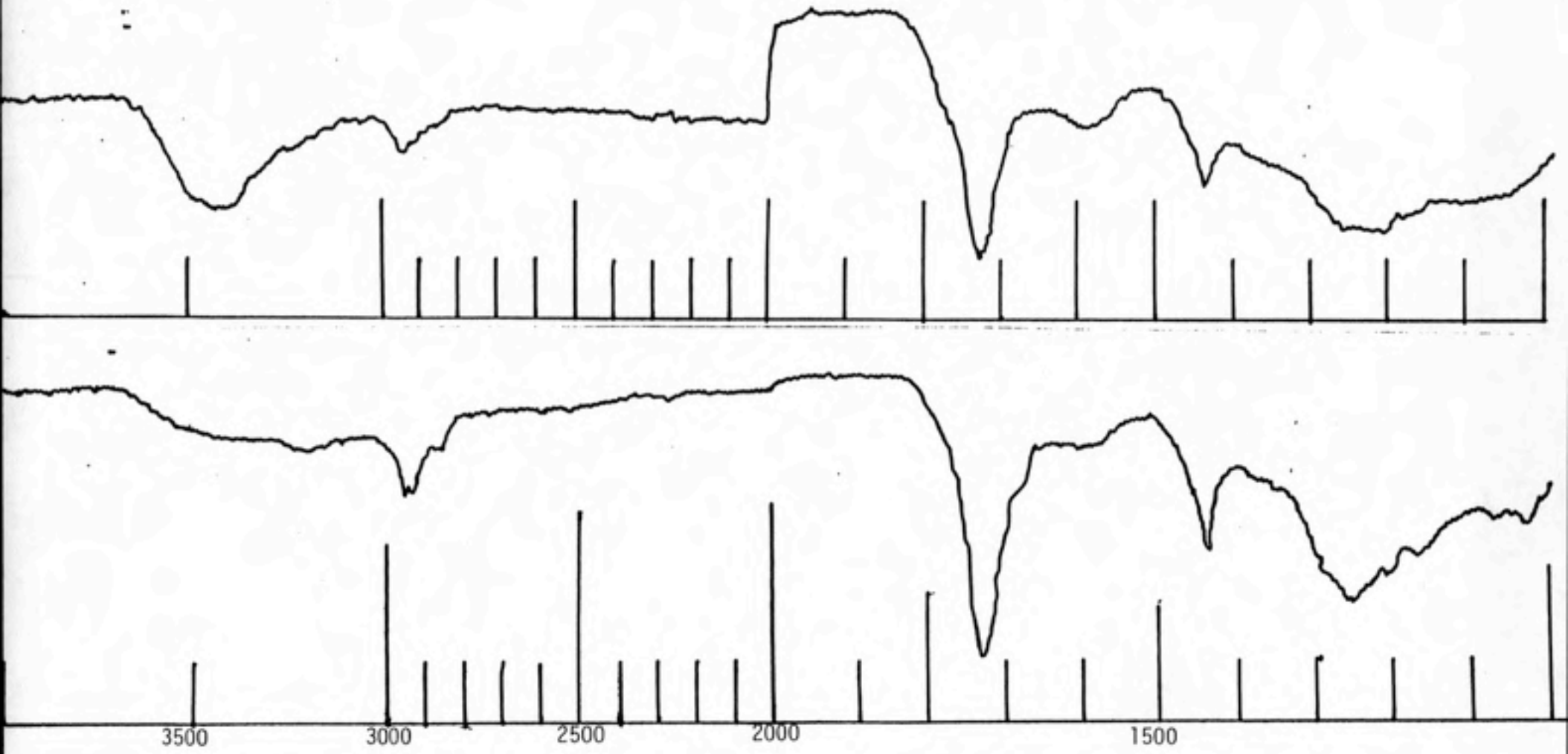
1.2.3 Methylation

Two separate procedures were used for methylation of fulvic acid. The first involved diazomethane generation using N-methyl-N-nitroso-N'-nitroguanidine (Aldrich Chemical) (ref.4). Once generated the diazomethane in ether was added and allowed to react with the fulvic acid. Several different ratios of diazomethane in ether and fulvic acid were tried and it was soon discovered that it required about 1 mL diazomethane/ether (concentration below) added to approximately 1 mg fulvic acid and a reaction time of 1 hour for complete methylation. After which the fulvic acid was blown dry N_2 at 45° C and an IR spectrum run (KBr pellet) to show complete methylation had taken place (refer to fig. 5).

Diazomethane/ether is generated by placing 1 mmole of N-methyl-N-nitroso-N'-nitroguanidine inside the inner tube of an apparatus that will capture the diazomethane gas in an ether collection tube. To the N-methyl-N-nitroso-N'-nitroguanidine 0.5 ml. of de-ionized/distilled water is added. The is tube capped with a rubber septum, and placed inside the outer tube containing 3 mL of ethyl ether. The apparatus is clamped shut and placed in an ice bath to cool the reaction. Using a syringe 0.6 ml of chilled 5M NaOH solution is added to the N-methyl-N-nitroso-N'-nitroguanidine in water. This is done under a protective hood using caution because if gas formation is too rapid the apparatus could explode. The reaction is allowed to proceed for 45 minutes after which the diazomethane/ether is removed and ready for use. If stored the solution, now a dark yellow, should be capped securely and kept refrigerated. This process should yield about 0.8 mmole diazo-

(top) Figure 5. I.R. Spectrum, Diazomethane Methylation of Fulvic Acid (SL104)

(bottom) Figure 6. I.R. Spectrum, BF3/MeOH Methylation of Fulvic Acid (SL104)



methane in 3 ml ether (ref.4).

The second methylation procedure used 14% BF_3 in Methanol (Aldrich Chemical). Approximately 2 mg of fulvic acid was added to 4 ml of 14% BF_3 / Methanol in a teflon-lined screw cap test tube. The tube was capped, vortexed, and heated to 95°C + or - 5°C for five minutes. The reaction mixture was cooled and the "methylated fulvic acid" extracted by adding 60 mL of H_2O, pouring the solution into a separatory funnel and extracting three times with 60 mL fractions of ethyl ether. The ether extract was dried over anhydrous sodium sulfate, filtered through glass wool and roto-evaporated to dryness. An IR spectrum of this methylated fulvic acid was also taken (fig.6).

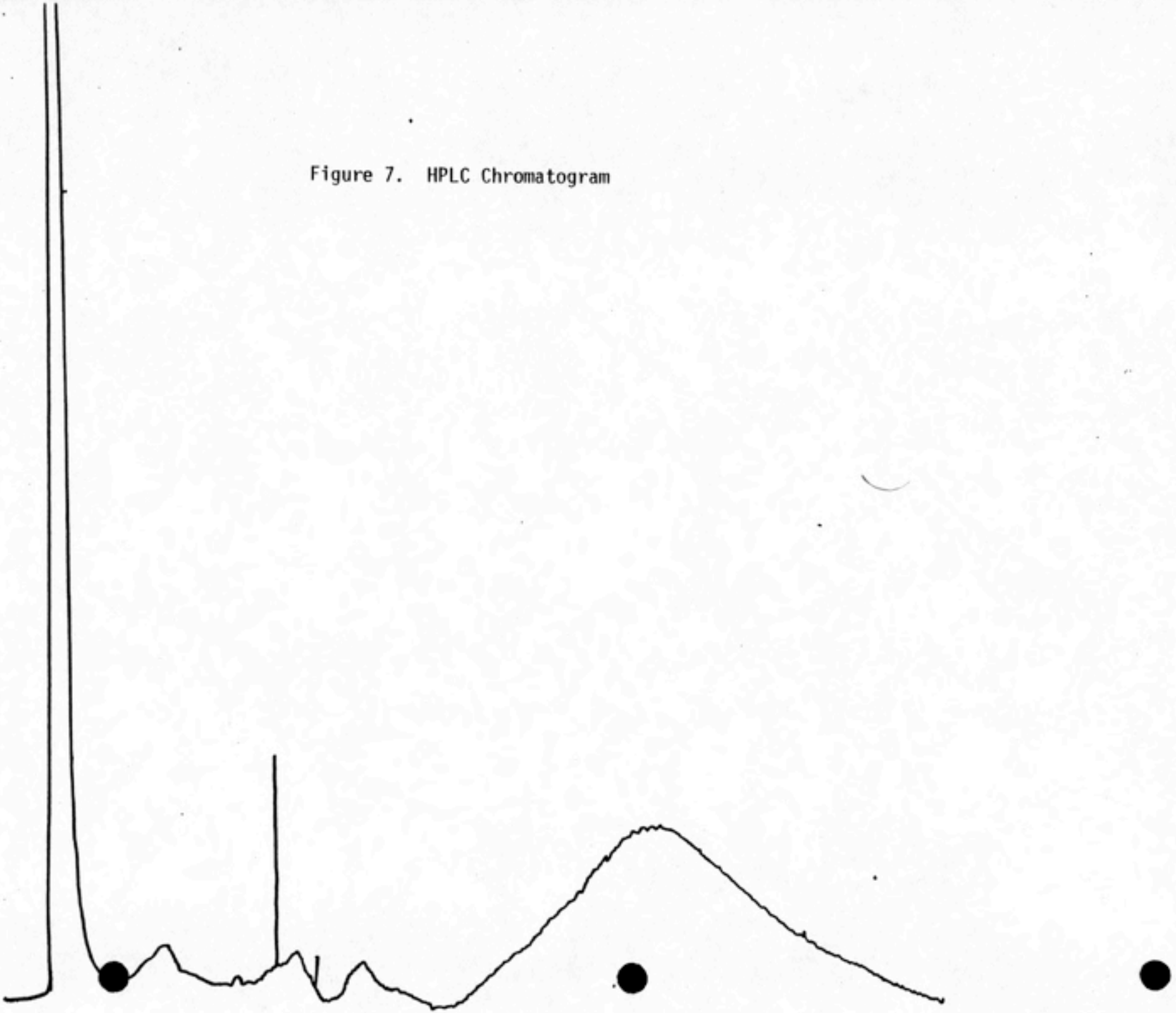
1.2.4 HPLC

In hopes of being able to identify other fractions of fulvic acid an HPLC separation method was attempted using methylated Aldrich fulvic acid. The first crude procedure produced some separation. (Refer to fig.7 and table 1 for the chromatograph and run conditions.)

TABLE 1 HPLC Run Conditions

Methylated Fulvic Acid: BF3/CH3OH			
Flow Rate: 2 mL/min			
Temperature: 35			
Pressure: 1116			
Column: S5CN, 4. 6X250			
Detector: UV 260 0.1 ODFS			
Mobile Phase Hex: MTBE: MeOH			
	Time	MTBE	MeOH
	0.0	100.0	0.0
	20.0	20.0	80.0

Figure 7. HPLC Chromatogram



1.2.5 NMR

Nuclear Magnetic Resonance (NMR) spectroscopy was also performed with methylated fulvic acid obtained from the humics group at UNC , batch SL104, and methylated with diazomethane. A ^{13}C NMR spectrum was run as well as a proton NMR spectrum. Figures 8 and 9 show the spectra and table 2 presents run conditions.

TABLE 2 NMR Run Conditions
Methylated Fulvic Acid

	Proton	13C
Pulse Width	3.00 usec	15.00 usec
	39 Degrees	76 Degrees
ACQ. Time	1.36 sec	229.38 msec
Recyle Time	8.15 sec	9.83 sec
No. of ACQS.	120	202,700
Data Size	16384	8192
Line Broadening	0.50 Hz	0.50 Hz
Spin Rate	14 RPS	12 RPS
Frequency	300.151850 MHz	75.480819 MHz
Spec Width	6024 Hz	17857 Hz
Plot Scale		
From	5.79	222.71
To	-0.80 PPM	-13.8 PPM

1.3 RESULTS AND DISCUSSION

1.3.1 Extraction of Fulvic Acid from Aldrich Humic Acid Sodium Salt

The IR spectra of the three collected Aldrich fulvic acid fractions (the non-dialyzed, dialysis-in and dialysis-out fractions; figures 1, 3 and 4 respectively) were compared to an IR spectrum of a Singletary Lake fulvic acid fraction (SL104) obtained from the Humics Group at the University of North Carolina (fig.2). These spectra show no distinguishable difference between the non-

Figure 8. ^{13}C NMR

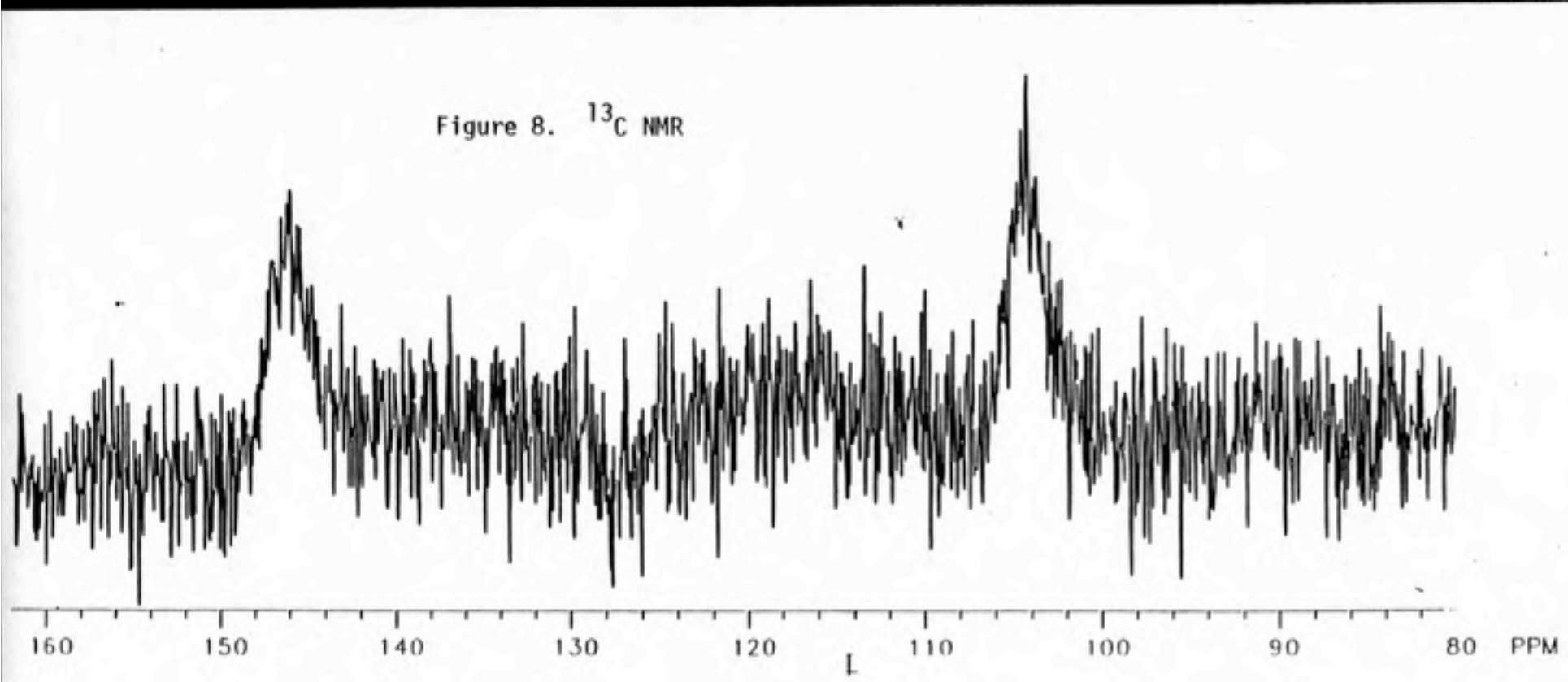
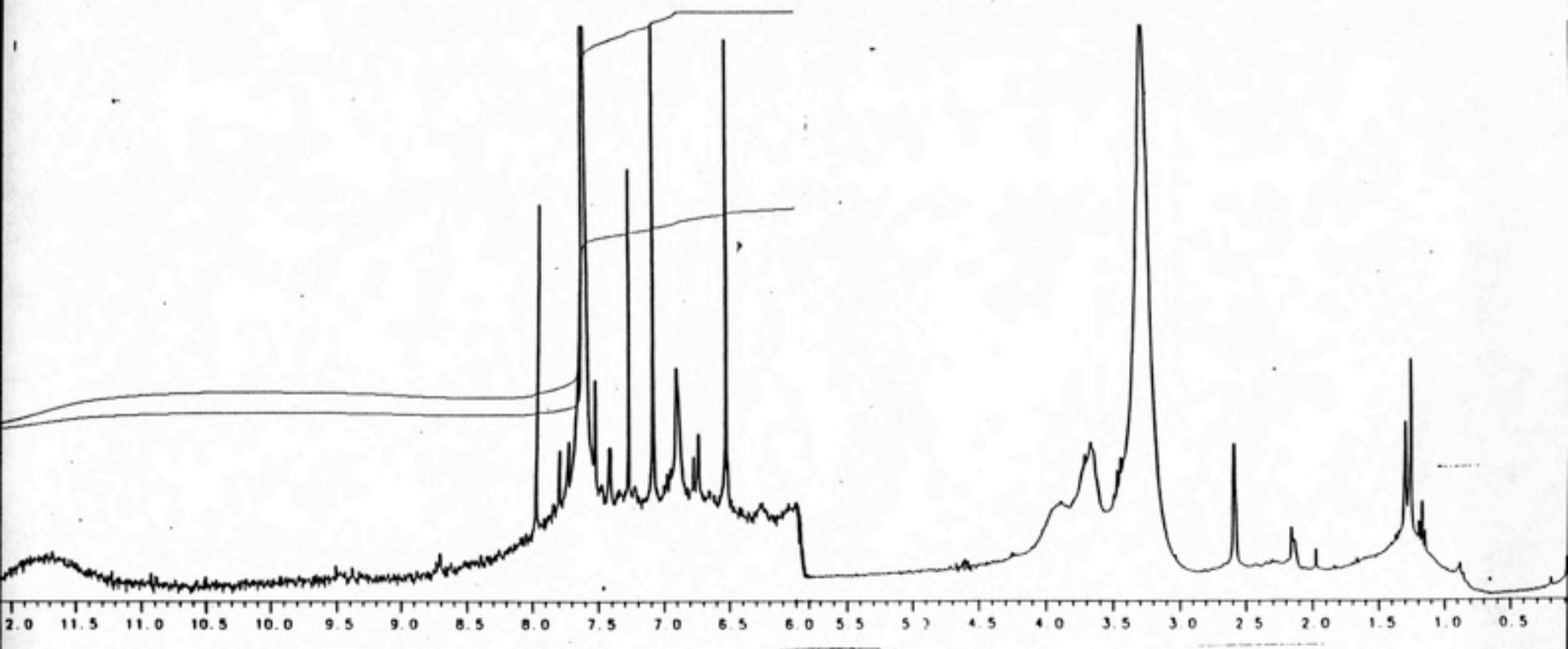


Figure 9. Proton NMR



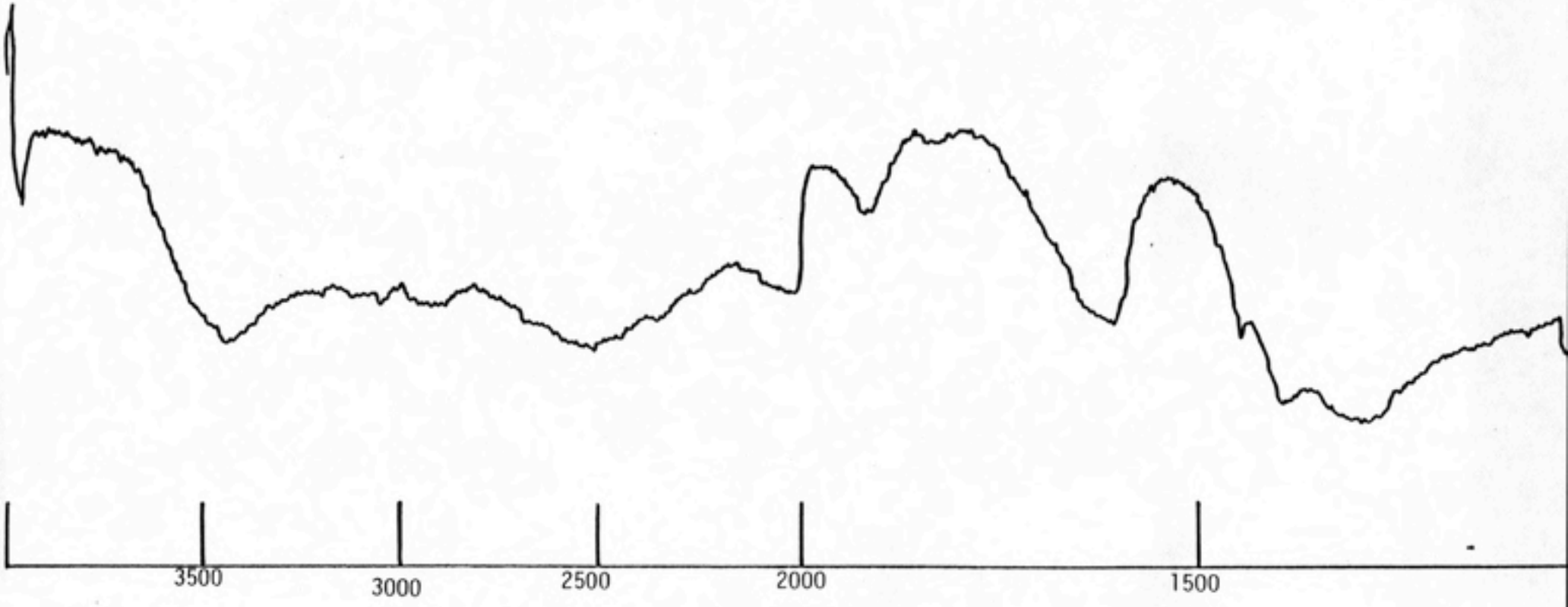
dialyzed and dialysis-in fractions of Aldrich fulvic acid. Any differences seen between these two fractions and the Singletary Lake fulvic acid tells us nothing of structural differences between the complexes. While Aldrich fulvic acid is obtained from a soil source and Singletary Lake fulvic acid is obtained from an aquatic source, and it is believed there are major structural differences between the two (ref.5), the IR spectra are of little use in distinguishing these differences.

Figure 4, the dialysis-out fraction, is an unusual spectrum for any previously examined fulvic acid spectra (ref.6). It shows very distinct peaks and was found to be very reproducible each time Aldrich fulvic acid was dialyzed. At first it was believed to be promising evidence for positively identifying a specific fraction of fulvic acid. However, by chance, this exact IR spectrum was discovered to be simply a spectrum of sodium bicarbonate (fig.10). Evidently sodium bicarbonate was artificially produced, presumably in the neutralization step following elution from the XAD-2 resin column. (Refer to the experimental section, 1.2.1.) In fact separation by dialysis showed that a majority (approximately 80%) of what was thought to be fulvic acid was simply sodium bicarbonate. However this spectrum gives evidence that fulvic acid will not dialyze through a dialysis bag with a molecular weight cutoff of 6,000-8,000. The IR spectrum of the dialysis-out fraction shows no indication of anything but sodium bicarbonate. (Compare figs. 4 and 10.)

1.3.2 Ash Content

The results of the ash content analysis are shown in Table 3.

Figure 10. I.R. Spectrum , Sodium Bicarbonate



The results were variable and much higher than I expected or desired. Since I was only able to characterize a small portion of fulvic acid and I was much more interested in Singletary Lake Fulvic acid I had little use for the ash content analysis done on my other fulvic acid fractions. An elemental analysis done by Micro-Tech laboratories of batch SL104 of Singletary Lake fulvic acid shows ash content to be very low (about 1%). Differences obtained by my experimental procedure and those obtained by Micro-Tech laboratories are because my fulvic acid is obtained as sodium salt, not as a free acid.

TABLE 3. ASH CONTENT OF FULVIC ACID

fulvic acid	Percent Ash Content (std .dev.)
Singletary Lake (SL105)	20 (11)
Aldrich (non-dialyzed)	31 (6)
Aldrich (dialysis-out)	34
*Aldrich (dialysis-in)	16 (6)

*Computed from dialysis-out and non-dialyzed fractions, not experimental.

1.3.3 Methylation

The IR spectra of the two methylated fulvic acid acid samples, diazomethane figure 5, and BF_3/Methanol figure 6, gave some valuable information concerning methylation. Evidence of complete methylation is indicated by disappearance of any carboxyl peak between 2700 cm-1 and 2500 cm-1 and the emergence of a sharp, large peak at 1732 cm-1. This new peak at 1732 cm-1 is the result of the formation of a methyl ester group from what were previously carbox-

yl groups. Both the diazomethane (ref.4) and BF_3 /Methanol methylation procedures have been shown to be carboxyl specific with interferences from phenol not found in our sample.

While the BF_3 /Methanol IR spectrum indicated incomplete methylation (fig. 6) the diazomethane methylation appeared complete when using the free acid form of fulvic acid (fig. 5). If any of the fulvic acid is in the salt form, methylation will not be complete since the mechanism for methylation requires the free-acid configuration (ref.4). The diazomethane methylation procedure also required a large excess of diazomethane/ether because of the insolubility of fulvic acid in ether. (Proportions used were stated in the previous paragraph.) This was used as the procedure of choice for future methylation.

1.3.4 HPLC

Methylated fulvic acid, run on HPLC, showed promising separation (fig.7). Table 1 shows run condition and peak areas recorded. I did not however continue with this investigation and chose another path for my research. I have included the chromatogram as an indication that HPLC separation may lead to a better characterization of fulvic acid. My research was directed toward functional group characterization and binding properties associated with aquatic fulvic acid and did not explore further separation by HPLC.

1.3.5 NMR

Nuclear Magnetic Resonance (NMR) Spectroscopy was valuable in developing a method for micro-quantitation of carboxyl groups in

fulvic acid (see section 3). Preliminary research with NMR indicated what could and could not be accomplished using fulvic acid. Dissolution of fulvic acid (Singletary Lake fraction) in D_2O showed no peaks of interest in either ^{13}C or proton NMR. The NMR operators were never successful in decoupling the water peak which appeared at full scale causing all other peaks to be insignificant. This water was not removable by freeze-drying, and from the IR spectra appeared to be either water of crystallization or some other tightly associated bound form of water. However, NMR spectra of methylated fulvic acid dissolved in a mixture of $CDCl_3$ /DMSO showed some interesting results in ^{13}C and proton NMR (figs.8 and 9 respectively). These spectra may be of further interest to those using NMR for characterization of fulvic acid, My purpose was to explore the possibility of NMR characterization of fulvic acid and present my findings. Interpretation of these spectra is left to the reader.

1.4 CONCLUSIONS

While my research branched into different directions I found these preliminary studies to be of use in gaining a better understanding of the material I was working with. These studies helped to define a direction from which my research could proceed. With so little known about fulvic acid and so much yet to be learned these attempts of defining possible research directions seemed appropriate. This however may be a somewhat confusing presentation and leave many questions yet to be answered. I included this information because I felt it may be useful to future research and because I found some of it to be beneficial to my other studies.

I realize this work represents incomplete experimentation and is not intended to constitute a research report. The rest of my report represents work in the development of specific procedures used in characterizing fulvic acid as well as some interesting results concerning binding properties. The three following sections deal with three separate and more complete phases of study.

REFERENCES

1) E.M. Thurman and R.L. Malcolm, Environ. Sci. Technol. 15, 463-466 (1981).

2) G. Ogner and M. Schnitzer, Science, 170, 317-318 (1970).

3) R.F. Christman, W.T. Liao, D.S. Millington, J.D. Johnson; "Oxidative Degradation of Aquatic Humic Material" in L.H. Keith, ed., Advances in the Identification and Analysis of Organic Pollutants in Water, Vol. 2, Chapt. 49, Ann Arbor Sci. Pub., (1981) pp. 979-999.

4) McKay, J. Am. Chem. Soc., 70, 1974 (1948).

5) E.T. Gjessing, Physical and Chemical Characteristics of Aquatic Humus, Ann Arbor Sci. Pub., (1976) pp. 15-85.

6) K.M. Goh and F.J. Stevenson, Soil Sci., 112, 392-400 (1971).

SELECTED METHODS FOR FUNCTIONAL GROUP ANALYSIS OF FULVIC ACID

by

Joseph Evans and Phillip W. Albro

National Institute of Environmental Health Sciences

Laboratory of Molecular Biophysics

PO Box 12233

Research Triangle Park, NC 27709

STRACT

Methods were evaluated for quantification of various functional groups in fulvic acid. These functional groups included aliphatic hydroxyls, phenolic hydroxyls, quinones, primary amines and bound carbohydrates. All tests performed required 1 mg or less of fulvic acid and yielded statistically reproducible results. Most functional groups were evaluated by two independent methods of differing specificities. Separate collections of fulvic acid were used for comparison.

INTRODUCTION

Fulvic acids are those organic compounds of natural waters that are not extractable into organic solvents and which do not precipitate at pH 1, Thurman and Malcolm (1981). Characterization of fulvic acid usually includes quantitative functional group analyses of the complex mixture of organic compounds present. Characterization of functional groups in fulvic acid preparations is useful in elucidating their composition and structure as well as determining differences between fulvic acids from various sources. While some investigators have used more traditional functional group determination procedures requiring large quantities of material, Schnitzer and Gupta (1964, 1965), Dubach and Menta (1963), others have used infrared spectroscopy,(Eltantaway and Bauctez (1978), Gjessing (1976), Goh and Stivanson (1971), Mathur (1972), Sipes and Sipos (1979), Wagner and Stevenson (1965)) which can often be qualitatively misleading and usually does not permit quantitation. Nuclear magnetic resonance (NMR) spectra can also be quantitatively misleading since most of the protons in fulvic acid are exchangeable. Hence various procedures have shown incomparability of results.

We therefore have selected what we believe to be some simple, functional group tests not previously used in fulvic acid studies. None of the methods presented are new, but are variations of previously published techniques used for other complex materials (e.g. proteins). Our presented methods require only small amounts of fulvic acid for accurate determinations. This would allow one to easily quantify functional groups from various fulvic acid samples. We have evaluated procedures for the following functional groups; total hydroxyls, phenolic hydroxyls, quinones, primary amines and bound carbohydrates. The fulvic

id we used was collected from Singletary Lake, N.C. by the Humics Group at the University of North Carolina at Chapel Hill, and extracted by a method similar to that of Thurman and Malcolm (1981).

EXPERIMENTAL

A) Total Hydroxyls

Weigh out approximately 1 mg of fulvic acid into a 15 ml screw top test tube with a teflon lined cap. Dissolve in 3 ml of redistilled, dry pyridine (Fisher, A.C.S.; Fair Lawn, N.J.) and add 1 ml of redistilled acetic anhydride (Baker Chemical; Phillipsburg, N.J.). Cap, and shake gently for 24 hours at room temperature. As reported by Petersen et al. (1973), this procedure will acylate all primary and secondary hydroxyl groups; even those that are sterically hindered.

After 24 hours the pyridine and excess acetic anhydride are blown off under N_2 at 60°C. A blank containing only acetic anhydride and pyridine is run as a control to check for complete evaporation of the acetic anhydride. Even small traces will interfere with the next step of the procedure.

The acylated fulvic acid is dissolved in 0.1 ml of 95% ethanol and the ferric hydroxamate procedure outlined by Rapport and Alonzo (1955) for spectrophotometric acyl ester determination is followed. A control experiment using non-acylated fulvic acid and following the procedure above is also done and a standard curve using amyl acetate (Fisher) is prepared. Finally a non-acylated fulvic acid sample is dissolved in 95% ethanol but not treated with the hydroxylamine or ferric perchlorate reagents. This is done since there is some absorbance by fulvic acid at a wavelength of 530 nm as used in the Rapport and Alonzo method. The concentration of this control should be similar to the sample run. all samples and controls are read against the same reagent blank.

Hydroxyl determination is extrapolated using the amyl acetate curve converting equivalents of acyl ester to equivalents of hydroxyl. A net O.D. for the sample is computed by subtracting the O.D. of the fulvic acid in ethanol only and the O.D. of the processed, non-acylated fulvic acid control from the O.D. of the acylated fulvic acid sample.

B) Phenolic Hydroxyls and Quinones

A similar detection method was used to determine both phenolic hydroxyls and quinones. For phenolic determination, the most specific method of those tested was that of Chrastil (1975) using 1 ml of a 1 mg/ml solution of fulvic acid in water. These results were compared to those obtained using the Folin and Denis phenol method. It was necessary to use a fulvic acid control of similar concentration without the $(NH_4)_2\ Ce(NO_3)_6$ added (Chrastil procedure) or the Folin phenol reagent added (Folin method) read against a reagent blank, and subtracting the O.D. of this control from that of the sample to obtain a net O.D. The results were extrapolated from a standard curve made using (p-hydroxyphenyl) propionic acid (Allied Chemical).

For quinones the fulvic acid was first reduced by adding 1 ml of a 10 mg/ml solution of $NaBH_4$ in 95% ethanol (Fisher Scientific Co.) to 1 ml of a 1 mg/ml solution of fulvic acid, allowing to react for 30 min., then adding a few drops of glacial acetic acid (Mallinckrodt; Paris, Ky.) to decompose any excess $NaBH_4$. Use the Chrastil Method for phenolic determination, computing a net O.D. as before with a reduced fulvic acid control with no $(NH_4)_2\ Ce(NO_3)_2$ added, extrapolating from the same standard curve of (p-hydroxyphenyl) propionic acid and converting phenolic hydroxyl equivalents to quinone equivalents. This value is corrected by subtracting any phenolic hydroxyl found prior to reduction with $NABH_4$.

Primary Amines

Two independent methods were used for comparison. The first uses fluorescamine (Roche Diagnostics; Nutley, N.J.,). Three mg of fluorescamine is dissolved in 10 ml of acetone. A 0.05 M phosphate buffer is made at a pH of 8.0. Ethanolamine (New England Nuclear; Boston, MA) is used as a standard, 40 nm/ml.

To duplicate standards and samples (fulvic acid 0.1 mg/ml) of 0.5 ml add 1.0 ml of the phosphate buffer. While vortexing add 0.5 ml of the fluorescamine solution. Let stand 30 minutes and read fluorescence in a fluorometer (Perkin-Elmer MPF-36) with 6 nm slits, a 390 nm excitation wavelength and a 475 nm emmission wavelength. The fluorometer should be standardized with a quinine solution and zeroed with the phosphate buffer. Fluorescence should be corrected using a fluorescamine blank; however, no fulvic acid control is needed.

The other method described by Mokrasch (1967), uses 2,4,6-trinitro benzene sulfonic acid (Eastman; Rochester, N.Y.). Use 0.5 ml of a 1 mg/ml fulvic acid solution following the aforementioned procedure. However, quench with ice-cold 88% formic acid (Baker Analysed rgt.) instead of methanol, reading against a reagent blank at 366 nm. No standard is needed; instead use an extinction coefficient of 1.2×10^4 M^{-1} cm^{-1}, Mokrasch (1967). We checked this figure with an Ethanolamine standard and calculated identical results. A fulvic acid control is needed, using a similar fulvic acid concentration, without adding the trinitrobenzene sulfonic acid. This is also read against a reagent blank and the O.D. of the control is subtracted from that of the sample to obtain a net O.D. Beer's Law ($A = \varepsilon cb$) is used [where A = absorbance (O.D), ε = extinction coefficient, c = molar concentration, b = length of cell] to calculate the molar concentration of amines.

b. Carbohydrates

For calculating bound carbohydrates two independent procedures were used. The first is an Anthrone reaction by Shields and Burnett (1960) using 1 ml of a 1 mg/ml. Fulvic acid solution. Net O.D. is computed, subtracting from the sample a fulvic acid control of similar concentration (no Anthrone rgt. added). This net O.D. is converted to percent bound carbohydrate using a standard curve.

The second method using α-naphthol as described by Dische (1955), was compared to the first. It required 0.5 ml of a 1 mg/ml fulvic acid solution. An appropriate curve was generated using a sucrose standard and net O.D. of the fulvic acid was once again corrected for with a fulvic acid control, of similar concentration, read against a reagent blank.

RESULTS AND DISCUSSION

Table 1 shows the results for the various functional groups obtained from the same fulvic acid sample. These results can be compared to results reported for other fulvic and humic acids, see Gjessing (1976).

We believe one of the more interesting results obtained in our experimentation was the indetectably low level of phenolic -OH in our fulvic acid sample using the Chrastil (1975) method. This differs from the Folin-phenol reagent results, which were positive for phenolic hydroxyls. However it is likely there are compounds in our fulvic acid sample which give a false positive with the Folin-phenol reagent. Several potentially interfering compounds are listed in papers by Folin and Denis, and Gutner and Holm. We believe benzaldehydes are possible in our fulvic acid which could cause interference as suggested by Folin and Denis. The procedure by Chrastil reportedly is much less subject to false positives from interfering compounds. Furthermore our sample showed positive results for quinones, which were reduced to phenolic hydroxyls, when the Chrastil procedure was used. The addition of unreduced fulvic acid to solutions of p-hydroxyphenyl propionic acid had no effect on the O.D. in the Chrastil procedure.

The amine results showed excellent correlation between the two independent methods used. This positive result for amines is helpful because fulvic acid can now be easily "tagged" with radioactive or fluorescent functionalities. Fluorescamine, for example, can be used for those purposes and is promising for HPLC work.

The results for total hydroxyls was comparable to results obtained by others. The bound carbohydrate results showed good correlation between the two

methods used. All our tests were designed so comparisons could be made readily on different batches of fulvic acid without using much sample. This will perhaps help those who are interested in batch to batch comparisons of various functional groups.

TABLE 1. Results obtained for functional group determination of aquatic fulvic acid from Singletary Lake, N.C.

Functional group	meq/gm fulvic acid	weight %
primary & secondary hydroxyls	2.75 ± 0.55	4.7 ± 0.9
phenolic hydroxyls		
(Folin phenol rgt.)	0.9	1.5
phenolic hydroxyls (Chrastil)	negative ($<5 \times 10^{-5}$ eq./gm)	negative
quinones	0.196 ± .004	-
primary amines (Fluorescamine)	0.097 ± .006	0.16 ± 0.01
primary amines (2,4,6-trinitrobenzene sulfonate)	0.094 ± .005	0.15 ± 0.01
bound carbohydrate (Anthrone method)	-	5.5 ± 0.7
bound carbohydrate (Dische)	-	4.2 ± 0.6

REFERENCES

Butler J.H.A. and Ladd J.N. (1969) Effect of extractant and molecular size on the optical and chemical properties of soil humic acid. Aust. J. Soil Res. 7, 224-277.

Chrastil J. (1975) Colorimetric estimation of phenols and tyrosine. Anal. Chem. 47 (13), 2293-2296.

Dische Z. (1955) New Color Reactions for Determination of Sugars, in Polysaccharides, in Methods of Biochemical Analysis, Vol. II, D. Glick (Ed.), Interscience, N.Y., pp. 313-358.

Dubach P. and Mehta N.C. (1963) The chemistry of soil humic substances. Soils and Fertilizers 25, 293-300.

Eltantawy I.M. and Bauctez M. (1978) Structural study of humic acids by x-ray, electron spin resonance, and infrared spectroscopy. Soil Sci. Soc. Amer. J. 42, 903-905.

Folin O. and Denis W. (1912) On phosphotangstic-phosphomolybdic compounds as color reagents. J. Biol. Chem. 7 (2), 239-243.

Gjessing E.T. (1976) Physical and chemical characteristics of aquatic humus. Ann Arbor Science, Inc., pp. 15-85.

Goh K.M. and Stivanson F.J. (1971) Comparison of infrared spectra of synthetic and natural humic and fulvic acids. Soil Sci. 112, 392-400.

Gortner R.A. and Holm G.E. (1920) The colorimetric estimation of tyrosine by the method of Folin and Denis. J. Biol. Chem. 40, 1678-1692.

Haworth R.D. (1971) The chemical nature of humic acids. Soil Sci. 111, 71-79.

Mathur S.P. (1972) Infrared evidence of quinones in soil humus. Soil Sci. 113, 136-139.

Mokrasch L.C. (1967) Use of 2,4,6-trinitrobenzene sulfonic acid for the coesti-

nations of amines, amino acids and proteins in mixtures. Anal. Biochem 18, 64-71.

Petersen J.W., Hedberg K.W. and Christensen B.E. (1943) Microdetermination of hydroxyl content of organic compounds. Ind. Eng. Chem. 15, 225-226.

Rapport M.M. and Alonzo N. (1955) Photometric determination of fatty acids ester groups in phospholipids. J. Biol. Chem. 217, 193-198.

Schnitzer M. and Gupta V.C. (1964) Some chemical characteristics of the organic matter extracted from the O and BZ horizons of a gray wooded woil. Soil Sci. Soc. Amer. Proc. 28, 374-377.

Schnitzer M. and Gupta V.C. (1965) Determination of acidity in soil organic matter. Soil Sci. Soc. Amer. Proc. 29, 274-277.

Shields R. and Burnett W. (1960) Detrmination of protein bound carbohydrate in serum by a modified anthrone method. Anal. Chem. 32 (7), 885-886.

Sipos S. and Sipos E. (1979) Infrared spectroscopic examination of humic acids. Acta Physica Chemica 25, 187-193.

Thurman E.M. and Malcolm R.L. (1981) Preparative isolation of aquatic humic substances. Environ. Sci. Technol. 15 (4), 463-466.

Wagner G.H. and Stevenson F.J. (1965) Structural arrangements of functional groups in soil humic acid as revealed by infrared analysis. Soil Sci. Soc. Amer. Proc. 29, 43-48.

MICRODETERMINATION OF CARBOXYL GROUPS IN FULVIC ACID AND RELATED POLYCARBOXYLATES

by

Joseph Evans and Phillip W. Albro*

Laboratory of Molecular Biophysics

National Institute of Environmental Health Sciences

PO Box 12233

Research Triangle Park, NC 27709

*Author to whom correspondence should be addressed

ABSTRACT

A radiochemical procedure was used to quantify carboxyl content of fulvic acid. Tritium from tritiated water exchanged with carboxyl protons in fulvic acid and were then locked into the fulvic acid structure by diazomethane methylation. Liquid scintillation counting of the exchanged methylated fulvic acid yielded quantifiable results using 100 microgram quantities of fulvic acid. Values obtained were comparable within 2% to those obtained a $Ca(OAc)_2$ titration procedure for carboxyl determination requiring 50 milligrams of polycarboxylate.

Key words: Fulvic Acid, carboxyl groups, radioassay, quantification

INTRODUCTION

Humic and fulvic acids are defined as those organic components of natural waters that are not extractable into organic solvents; the former precipitates at pH 1 while the latter does not (1). During studies of the binding properties of fulvic acid from different sources we found it necessary to compare small batches as to carboxyl group content. Standard methods for this determination exist (2,3), but require relatively large amounts of material (on the order of 50 mg, ref. 3). As the purification of even mg amounts of fulvic acid is time consuming and tedious (1), we needed a method for carboxyl group determination applicable to submilligram quantities of polycarboxylates. Since analyses for phenolic hydroxyl groups (6) had indicated that our preparations of fulvic acid were extremely low in this functionality, we decided to take advantage of the characteristics of the diazomethane esterification reaction as a means of radiochemical determination of carboxyl groups.

Diazomethane has been shown to introduce $-CH_2-$ groups into cyclic structures of humic or fulvic acid (4). For this reason we could not use radiolabeled diazomethane for carboxyl group determination. Preliminary experiments involving proton nuclear magnetic resonance spectroscopy in D_2O revealed that essentially all of the protons in fulvic acid that were capable of exchanging did so very rapidly, exchange being complete in less than 90 seconds. This being the case, radioactivity could be introduced and "fixed" as described below.

MATERIALS AND METHODS

Fulvic acid used in our experiments was collected from Singletary Lake and Black Lake, NC. It was isolated by the humics group at the University of North Carolina, Chapel Hill. The isolation procedure used was as described by Thurman and Malcolm (1).

a) Exchange; Esterification; Re-exchange

Start with duplicate samples containing 100 μg of fulvic acid in 100 μl of deionized water aliquoted into 0.5 dram glass vials. Benzene pentacarboxcylic acid (Pfaltz and Bauer; Stanford, Conn.) was used as a separate reference standard in each set of samples to correct for variations in dilution of ^3H_2O by moisture in the diethyl ether as well as humidity in the air. The samples and standards are placed in a vaccum desiccator over Drierite ($CaSO_4$, Hammond Co.; Xenia, Ohio) and NaOH pellets (Allied Chemical; Morristown, NJ) until dry (usually 24 hours).

To each of the dry samples and standards add 20 μl of ^3H_2O (activity used 2.49×10^8 DPM/g, obtained from New England Nuclear, Boston, MA) and vortex. Allow 2 minutes to assure complete exchange of tritium with hydrogen in the samples and then add 1 ml of diazomethane in ether previously generated as described by Fales et al. (5) using N-Methyl-N-nitro-N-nitrosoguanidine (Aldrich; Milwaukee, WI). The vials are securely capped with a teflon lined screw cap, and placed on a shaker for 1 hour.

Pure parafin (Gulfwax), 1 mg, is then added, only to the vials containing benzene pentacarboxylic acid to prevent formation of a dry dust. The samples and standards are dried at 38°C under a stream of N_2 for 10-20 minutes. To the now methylated samples one drop of non-labeled, de-ionized water is added, the

vials vortexed, and standards as well as samples are put back in the vacuum desiccator overnight. This step is repeated the following day.

b) Radioassay Procedure

To the dried samples and standards were added a few drops of methanol or acetonitrile-methanol (1:1, v/v) to dissolve the solid residue. Each small vial was then crushed, using a pair of pliers, inside a larger scintillation vial. To these were added 15 ml of Riaflour (New England Nuclear; Boston, MA) and the incorporated (non-exchangeable) tritium radioassayed using a liquid scintillation counter (Packard, Tri Carb 4530).

c) Calculation of Results

(1) The benzene pentacarboxcylic acid is used to determine the effective specific activity of the tritiated water by the following method:

$$\text{effective specific activity} \equiv \frac{\text{equiv. } COOCH_2{}^3H \text{ counted}}{\text{equiv. COOH originally present}}$$

$$\text{equiv. } COOCH_2{}^3H \equiv \frac{\text{DPM observed (benzene pentacarboxcylic acid)}}{\text{DPM/g (Activity of } {}^3H_2O \text{ used) x 18g/equiv. } {}^3H_2O \text{ x } 1/2}$$

*The specific activity per ^3H is 1/2 that of ^3H_2O hence the 1/2 used above.

$$\text{equiv. COOH originally present} \equiv \frac{\text{gm. benzene pentacarboxcylic acid}}{298} \times 5$$

*5 equiv. COOH per mol benzene pentacarboxcylic acid

**298 = F.W. of benzene pentacarboxcylic acid

(2) Equivalents of carboxyl in the fulvic acid samples is computed as follows:

$$\frac{\text{equiv. } COOCH_2{}^3H \text{ (counted in methylated fulvic acid)}}{\text{effective specific activity}}$$

$$\text{equiv. } COOCH_2{}^3H \text{ (counted in methylated fulvic acid)} \equiv \frac{\text{DPM observed (methylated fulvic acid)}}{\text{DPM/g (Activity of } {}^3H_2O \text{ used) x 18g/ea. } {}^3H_2O \text{ x } 1/2}$$

(3) Equivalents of carboxyl per gram of fulvic acid is then computed.

$$\frac{\text{equiv. COOH in sample}}{\text{gm. of fulvic acid}}$$

(3a) To obtain a weight percent of carboxyl in fulvic acid

$$\text{weight percent} \equiv \text{(equiv. COOH/gm. of fulvic acid) x 45 x 100}$$

*45 = F.W. of COOH

d) Control Experiments

To test the exchange of carboxyl protons with water we put a few milligrams of benzene pentacarboxcylic acid into two separate NMR tubes and to one added d_6-DMSO (Merck, Sharp & Dohme; Kirkland, Que. Canada) and to the other 100% D_2O (Aldrich; Milwaukee, WI). Noting the time when we dissolved our standard in D_2O would allow us to determine how much time is required for a complete exchange of hydrogen with deuterium by comparing the NMR spectra of both solutions. The NMR spectrum of benzene pentacarboxcylic acid in d_6 DMSO would show no exchange with deuterium while the D_2O NMR spectrum examined at several different time points would show exchange with respect to time. Used for all NMR spectra was a Nicolet QE 300, 300 MHz NMR spectrophotometer, 2.05 seconds/scan.

As indicated previously it is necessary to run a benzene pentacarboxcylic acid standard with each set of samples to determine an effective specific activity. To better understand why this varies from run to run we ran an experiment using benzene pentacarboxcylic acid. We exchanged the protons with D_2O instead of ^3H_2O, methylated our standard, dried it, and dissolved it in d_6-DMSO. By comparing the NMR spectrum of this sample with a benzene pentacarboxcylic acid sample that was methylated but not exchanged with D_2O we could determine what percentage of our methyl groups were $-CH_2D$ and if any $-CH_3$ groups still remained.

To evaluate the percentage esterification by our diazomethane methylation procedure we methylated, as previously described, benzene pentacarboxcylic acid samples and fulvic acid samples. These were dried, pressed into pellets with KBr (Perkin Elmer; Norwalk Conn.) and infrared spectra were run on a Perkin Elmer 1320 infrared spectrophotometer. These IR spectra were compared to those of non-methylated benzene pentacarboxcylic acid and fulvic acid samples.

Fulvic acid contains exchangeable protons other than carboxyl. This is why the re-exchange of tritium with hydrogen after methylation is necessary. To test for complete re-exchange of protons with groups other than carboxyl we used an hippuric acid (Fig. 2) standard which has an exchangeable proton attached to a nitrogen, not subject to methylation under these conditions, as well as having an exchangeable carboxyl proton. It was exchanged, methylated and re-exchanged as described for fulvic acid and radioassayed with a standard benzene pentacarboxcylic acid sample for comparison.

Finally to compare our method of carboxyl determination with a more standard method we chose to run one sample of fulvic acid both by the present method and by the procedure described by Schnitzer and Gupta (2).

RESULTS

The results of carboxyl determination by the tritium exchange procedure are shown for different samples of fulvic acid in table 1. The comparison of the results obtained for one sample of fulvic acid by the tritium exchange method versus the $Ca(OAc)_2$ method (2) is shown in table 2. As shown in the table the two separate methods gave similar results confirming the validity of our tritium exchange procedure. The $Ca(OAc)_2$ method required 50 mg of fulvic acid. The different samples of fulvic acid in table 1 were collected separately at various times during the year and show some batch to batch variation.

When we examined the proton NMR spectra of the two samples of benzene pentacarboxcylic acid dissolved in d_6-DMSO and D_2O respectively, our sample in DMSO showed two peaks as expected. The peak at 8.6 PPM (tetramethyl silane reference), reflected the single hydrogen on the benzene ring, and the other, a rather broad peak at 13.7 PPM, represented the collection of the 5 hydrogens on the carboxyl groups. The sample in D_2O showed the peak at 8.6 PPM but no peak at 13.7 PPM after only 1½ minutes, indicating complete exchange of hydrogen by deuterium on all 5 carboxyls. This confirmed the exchange was both rapid and complete.

The NMR spectrum of the benzene pentacarboxcylic acid exchanged with D_2O and then methylated, compared with the spectrum of methylated, non-exchanged benzene pentacarboxcylic acid clearly demonstrated the need to determine the "effective" specific activity of the tritiated water for each batch of samples processed. We knew from our previous results exchange is 100%; however as shown in Figure 1, since there is a slightly different shift for a $-CH_2D$ group from a $-CH_3$ group, 78% of our exchanged standard was $-CH_2D$ while the remaining fraction was $-CH_3$. This indicated re-exchange with hydrogen had taken place before

methylation. Evidently there is a variable dilution effect from run to run probably dependent upon the humidity in the lab air or trace amounts of water in the ether. This is why it is necessary to include a standard of known COOH composition with each sample run.

Complete esterification of our fulvic acid and benzene pentacarboxcylic acid was verified by infrared spectroscopy. The absence of any peak from 2650 to 2000 cm^{-1} (COOH region) in our methylated samples, as was previously present in our non-methylated samples, and the emergence of a large sharp peak at 1730 cm^{-1} (methyl ester carbonyl) in our methylated sample confirmed complete esterification in both the fulvic acid and benzene pentacarboxcylic acid (within the limits of detection of infrared spectroscopy, roughly 3% -COOH detectable).

Finally, our control experiment using hippuric acid to test for the complete re-exchange of tritium, other than carboxyl, gave results that correlated precisely with those recorded for benzene pentacarboxcylic acid. The effective fixation of ^{3}H in the methylated hippuric acid was 85.7% ± 1.7% and for benzene pentacarboxcylic acid 84.2% ± 2.5%. This confirmed the re-exchange procedure was complete.

*Note due to limited space IR and NMR spectra are not included. They are available upon written request.

DISCUSSION

In performing this assay it is important to use quantities similar to those stated. We found new problems occured when we scaled down or up from the presented quantities. For example, 100% methylation of the fulvic acid is achievable only when the diazomethane is available in excess, possibly because of competing side reactions. Incomplete methylation was easily observable in infrared spectra of samples containing too little diazomethane. The same problem can occur if too much tritiated water is used, causing two phases, ether and water. When this happens the fulvic acid remains in the water phase and the diazomethane in the ether phase, hence incomplete methylation. Reaction time and continuous shaking are also important to assure 100% methylation.

While this method is designed for fulvic or humic acid some researchers may find it useful for other polyanions as well. It was designed to be used when only small amounts of a high molecular weight organic material are available.

Table 1

COMPARISON OF CARBONYL CONTENT OF VARIOUS SAMPLES OF FULVIC ACID

Sample[a]	1	2	3	4
$\frac{\text{mequiv COOH}}{\text{gm. Fulvic Acid}}$	5.78 ±0.46	3.65 ±.16	- 4.00 ± .25	3.65 ±0.11
weight %	26.0 ± 2.1	15.4 ± 0.7	18.0 ± 1.1	16.4 ± 0.5

[a]Samples 1, 2 and 4 from Lake Singletary, sample 3 from Black Lake.

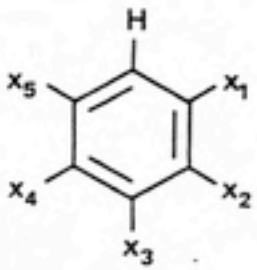
Table 2

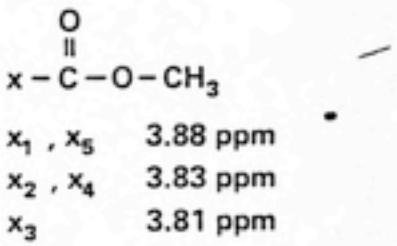
COMPARISON, BY TWO INDEPENDENT METHODS OF CARBOXYL CONTENT OF ONE FULVIC ACID SAMPLE

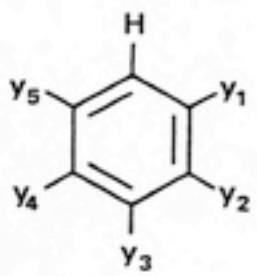
Fulvic Acid Sample 1

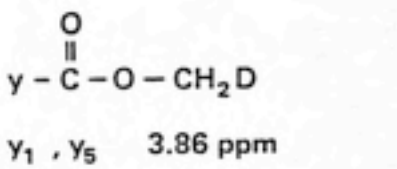
Method	Tritium exchange	$Ca(OAc)_2$ (4)
$\frac{\text{mequiv COOH}}{\text{gm Fulvic Acid}}$	5.78 ± 0.46	5.19 ± 0.13
weight %	26.0 ± 2.1	23.4 ± 0.6

Figure 1: Proton NMR Spectroscopy of Native and D_2O-exchanged Benzene Pentacarboxylate. The peak area ratios indicated a 78% trapping of ^2H in the methyl groups for this sample.









Comparison of NMR peak areas at 3.86 ppm to 3.88 ppm showed approximately 78% - CH_2D to 22% - CH_3.

References

1. E.M. Thurman and R.L. Malcolm, Environ. Sci. Technol. 15, 463-466 (1981).
2. M. Schnitzer and V.C. Gupta, Soil Sci. Soc. Amer. Proc. 39, 274-277 (1965).
3. L. Blom, L. Eddhauser and D.W. van Kreuelen, Feul 36, 135-153 (1957).
4. M. Schnitzer, Soil Sci. 117, 94-102 (1974).
5. H.M. Fales, T.M. Jaouni and J.F. Babashak, Anal. Chem. 45, 2302-2303 (1973).
6. J. Chrastil, Anal. Chem. 47, 2293-2296 (1975).

4.0 BINDING OF NON-POLAR ORGANIC POLLUTANTS TO FULVIC ACID

4.1 INTRODUCTION

Presented in the final section of this report are experiments involving interactions of non-polar organic pollutants with Aldrich and aquatic fulvic acid. The pollutants which were used are dimethyl phthalate (DMP), diethyl hexyl phthalate (DEHP), 2,3,7,8-TCDD (dioxin), DDT, and the herbicides, Amytryne and Prometone. These organics were chosen because there was evidence indicating that all but one, Dioxin, could bind to fulvic acid (ref.1-5). The original objective of this section was to determine binding constants for some of these organics and determine the number of possible binding sites on fulvic acid. For most of these compounds little is known about the nature of the binding mechanism and little evidence even exists to support binding to fulvic acid.

One report shows binding of phthalates to fulvic acid to be as high as four moles of phthalate to one mole of fulvic acid (ref.2). The binding mechanism was believed to be a combination of hydrophobic interactions and H-bonds. The herbicides Ametryne and Prometone have also been shown to bind to fulvic acid (ref. 3). The evidence showing binding of herbicides is greater than that for phthalates and the mechanism (ion-exchange) is better known. Other reports have also shown evidence of DDT binding (ref.4). To date no evidence could be found showing that Dioxin could bind to fulvic acid; however because so many other non-polar organic compounds were believed to be able to bind to fulvic acid it was postulated that

Dioxin could bind also.

The binding studies previously sited have led to a general belief that fulvic acid can transport many non-polar organics through the environment. This report however will question much of the evidence supporting binding or interactions of these compounds with fulvic acid. Each experiment was done in a progressive order so more could be learned about binding and about the mechanisms involved. DMP was used first because of its reportedly high binding affinity (ref.2) and because of its water solubility. If a binding constant could be determined, at different concentrations of pollutant and different concentrations of fulvic acid, more could be learned about the mechanisms involved. If DMP binding proved successful, DEHP would be used in the same manor, helping to gain further insight into hydrophobic or H-bond binding mechanisms. As will be seen in section 4.3 neither of these compounds were found to significantly bind to fulvic acid. Dioxin also showed no evidence of being able to bind.

The herbicides Ametryne and Prometone were used because more was known about their binding properties and because neither phthalates nor Dioxin could be shown to bind to fulvic acid. The results of herbicide binding proved to be inconclusive because of the difficulty in detecting bound pollutant. This led to the use of DDT as still another possible compound that could be used to test the validity of the experimental method used to separate bound and free pollutant (see section 4.2). These results left the researcher with still more questions on the binding of certain pollutants with aquatic or Aldrich fulvic acid. This research

raises new questions on whether or not non-polar organic compounds can bind to fulvic acid and suggests that the medium used for transport of these pollutants in the environment may be something other than humic material.

4.2 MATERIALS AND METHODS

This section includes the experimental conditions relating to various experiments on the binding of fulvic acid to non-polar organic pollutants. It is divided into 13 sub-sections and each sub-section has a matching section in the results and discussion. The experiments proceed in the order they were performed indicating a logical sequence of events as new and often surprising evidence was gained at the conclusion of each experiment.

4.2.1 Binding of Dimethyl Phthalate (DMP) to Fulvic Acid: Sep-Pak Procedure 1

Dimethyl phthalate (DMP) was used because of its water solubility and because previous evidence indicated binding to fulvic acid (ref.1,2). The solubility of DMP in water is very good for a non-polar organic compound (approximately 4mg/mL) and previous research had suggested the binding of DMP to fulvic acid to be as high as four moles DMP to one mole fulvic acid (ref.2). It was thought that if DMP binding could be quantitated, binding affinity could be determined for fulvic acid. ^{14}C labeled DMP (0.5 uCi/umol) was diluted with unlabeled DMP (Aldrich Chemical) to a concentration of approximately 3,500 DPMs/uL. This was done so an adequate amount of DMP could be used without the experimental sample being too radioactive and so the radio-labeled DMP could be conserved.

Five glass test tubes were prepared as follows:

-Tubes 1,2,and 3 contained 2.9 mL deionized/distilled water. Added to each of these tubes was 1.8 uL DMP (2.14 mg or 11 umol) and the tubes were then capped. The DMP was allowed to solubilize over a period of 48 hours of constant shaking.

-Next was added 0.1 mL of Singletary Lake fulvic acid (SL104) obtained from the humics group at UNC-CH. (its concentration in water was 5.1 mg/mL). If DMP was to bind to fulvic acid at an approximate ratio of 4 to 1 molar and if an average molecular weight for fulvic acid is assumed to be 1000 (ref.2) then approximately 20% of the DMP added should bind to the fulvic acid.

-This mixture was allowed to equilibrate for 24 hours.

Note: The 24 hour period stated for equilibrium in each experiment is not always 24 hours. It can actually mean 24 to 72 hours. There appeared to be no difference whether these equilibrations were allowed to proceed for 24 or 72 hours therefore no distinction was made in the written experimental procedures.

-Tubes 4 and 5 were run along with tubes 1,2 and 3 as blank controls. They were prepared similar to the other tubes however they contained no fulvic acid.

-Following equilibration $NaHCO_3$ (0.01M) was used to pH each solution to neutral (pH 7 to 8). At a neutral pH fulvic acid had less affinity for a C18 sep-pak cartridge. Further experimentation showed elution of fulvic acid to be incomplete and will be discussed in later sections.

Note: The blank solutions were not pH'd.

-Five C18 sep-pak cartridges (Waters) were pre-wetted, per recom-

mendation of the manufacturer, using 2 mL of methanol followed by 5 mL of de-ionized/distilled water. This step proved to be very important.

-The solutions containing the 3 fulvic acid samples and 2 blank controls, were poured into 5 separate 5cc syringes which had been previously attached to 5 respective sep-pak cartridges, labeled 1 through 5. The solutions were eluted at an approximate rate of 10 mL/min through the cartridges into 5 separate scintillation vials also labeled 1 through 5. An additional 1 mL of de-ionized/distilled water was passed through each sep-pak cartridge into the numbered scintillation vials. These were the water fractions of the respective solutions. (ref.5).

-Following this fraction were elutions of 3 mL of methanol followed by 1mL of methanol into 5 new scintillation vials. These elutions were labeled as the organic fractions of the respective solutions.

-Each sep-pak cartridge was broken open, its contents dumped into 5 separate scintillation vials, and labeled as the sep-pak fractions of solutions 1 through 5.

-There were in total 15 scintillation vials, to which 15 mL of Atomlight was added, shaken, and counted on a Packard liquid scintillation counter three times for five minutes. Each five minute count was then averaged and recorded as disintegrations per minute (DPMs).

4.2.2 Binding of Dimethyl Phthalate to Fulvic Acid (Sep-Pak, Procedure II)

To test whether varying the elution flow rate through the C18 cartridge would change the apparent binding affinity of DMP to

fulvic acid, three different flow rates were tested while the experimental conditions remained constant. ^{14}C labeled DMP at a concentration of approximately 28,000 DPM/uL was used adding only 1 uL (5.8 umol) DMP per tube. The DMP concentration to fulvic acid concentration was approximately 11 to 1 molar. If binding of DMP to fulvic acid is 4 to 1 molar, than 36% of the DMP (10,200 DPMs), would theoretically bind to the 0.5 umol of fulvic acid. (The DPM concentration was increased to allow for easier detection.)

Experimental conditions, except flow rate, were the same as section 4.2.1 and a total of 12 tubes were run. Six samples with fulvic acid (SL104), two tubes per flow rate tested (30mL/min, 10mL/min,and 1 mL/min), tubes 1,2; 3,4; and 5,6 respectively. Six blank tubes (no fulvic acid), were also run, tubes 7,8; 9,10; and 11,12 (30mL/min, 10ml/min, and 1mL/min, respectively). Following equilibration, pH, elution of the two fractions (water and organic); the three fractions (water, organic and sep-pak cartridge) were counted by liquid scintillation counting as in section 4.2.1.

4.2.3 Binding of Diethyl hexyl phthalate (DEHP) to Fulvic Acid, Sep-Pak Procedure

This experiment was intended to test binding affinity of DEHP to fulvic acid. ^{14}C labeled DEHP (10.7 uCi/umol) was diluted in hexane and used at an approximate concentration of 17,000 DPMs/uL. -Nine tubes were prepared. Tubes 1-3 were blank controls, tubes 4-6 were Singletary Lake fulvic acid (SL104) and tubes 7-9 were Aldrich fulvic acid. (Refer to section 1 for the preparation of

Aldrich fulvic acid.)

-Each tube received 9 uL DEHP in hexane (4.87 umol DEHP) and was then dried under N_2 at 60° C to evaporate the organic solvent.

-After evaporation, 2.9 mL of de-ionized /distilled water were added and the DEHP was allowed a few hours to solubilize. (DEHP is very insoluble in water and therefore only a small fraction was expected to dissolve.)

-Tubes 4 through 6 received 0.1 mL of fulvic acid (SL104) in water. (0.46 mg fulvic acid with an average molecular weight of 1,000 is 0.46 umol)

-Tubes 7 through 9 received 0.1 ml of Aldrich fulvic acid in water (0.51 mg fulvic acid or 0.51 umol).

Note: The molar ratio of DEHP to fulvic acid for tubes 4 through 9 was approximately 10 to 1, Binding was expected to be as high as 4 to 1 molar (ref.2), however, even if binding were only 1 to 1 molar, than 10% of the total DEHP added (15,000 DPMs) would bind to fulvic acid. If a detection limit of a few hundred DPMs is assumed any significant binding could be detectable.

-All tubes were capped and allowed to equilibrate with gentle agitation for 24 hours.

-Tubes 4 through 9 were then pH'd to neutral with 0.01M $NaCO_3$.

-All tubes were centrifuged for 15 minutes.

-After centrifugation the contents of each tube was pipetted, one by one, into a 5cc syringe and passed through a pre-wetted C18 sep-pak cartridge. This first elution was followed by 1 ml de-ionized/distilled water. These two elutions were combined and labeled as the water fraction.

-The next elution used 3 ml of methanol and was labeled as the

methanol fraction.

- The final elution used 3 mL of butanol and was labeled as the butanol fraction. This elution removed any remaining DEHP on the sep-pak therefore no sep-pak fraction was counted.
- All remaining counts were assumed to be left in the sample tube and a final rinse of the original sample tube was not done. Instead total DPMs were counted for 9uL of DEHP in hexane and were reported as total DPMs added. Experience had shown DEHP recovery to be not measurably less than 100%.
-Each fraction was counted, as was done in the DMP binding experiment. The three count average for each fraction was recorded.

4.2.4 Affinity of Fulvic Acid for C18 Sep-Pak Cartridges

A procedure, which quantitates organic carbon (ref.9), was used for determining the quantity of fulvic acid remaining on a sep-pak cartridge after water elution. While previous researchers had used C18 sep-pak cartridges for separating bound from un-bound pollutant (ref.5) there was no indication in their reports that fulvic acid had an affinity for these cartridges. The color of the cartridges following elution with water, and the color of the organic elution, indicated however some portion of the fulvic acid passed through the cartridge only after elution with an organic solvent.

- A reagent solution of 2.5 g $K_2Cr_2O_7$ (Fisher Scientific) dissolved in 1 L of 36N H_2SO_4 was prepared.
- Solutions of Aldrich fulvic acid and Singletary Lake fulvic acid (SL104) were prepared with concentrations similar to the DEHP

binding experiment.

- Duplicate 3 mL portions of each fulvic acid (Aldrich and Singletary Lake) were pH'd to neutral, and eluted through pre-wetted C18 sep-pak cartridges as was done in the binding experiments.
- This elution was followed by 1 mL of de-ionized/distilled water.
- These two elutions were combined and labeled as the water fraction.
- The other fraction (organic solvent) was a 3 mL methanol elution following the water elution.
- A water blank and a methanol blank (no fulvic acid) were also eluted through a C18 sep-pak cartridge. These fractions were appropriately labeled.
-It was assumed all fulvic acid was removed from the sep-pak after the methanol elution. This judgment was made because no color material remained on the sep-pak cartridge.
- To each fraction 1 mL of the $K_2Cr_2O_7$ solution was added, shaken, and heated to 100^{o} C for 45 minutes with occasional shaking.
- A 0.5 mL aliquot of each sample and blank control was taken. To this fraction 20 mL of water was added and each fraction was read at an absorbance of 350 nm, using water as a spectraphotometric blank.
- An organic carbon standard with 350 ug of carbon was prepared and run as a check standard for the procedure.
-Net absorbance (O.D.) was computed as follows:

Absorbance of water blank - absorbance of sample = net absorbance

4.2.5 Affinity of Fulvic Acid for XAD-2 Resin

The previous experiment had shown a significant amount of fulvic acid remained bound to the C18 sep-pak cartridge after eluting with water. To avoid this problem hand packed pasteur pipettes filled with soxhlet extracted XAD-2 resin (Supelco, Inc.) were used in place of C18 sep-paks. The same experiment was run as in section 4.2.4. Slight modifications included testing of Singletary Lake fulvic acid only and not Aldrich fulvic acid, and no organic carbon standard was tested.

4.2.6 Binding of Diethyl hexyl phthalate (DEHP) to Fulvic Acid: XAD-2 Resin Procedure

This experiment was a repeat of section 4.2.3 , however, hand packed XAD-2 resin columns were used in place of C18 sep-pak Cartridges. Hand packing consisted of placing a small piece of glass wool inside a pasteur pipette, assuring XAD-2 resin would remain inside the column, followed by filling the pipette approximately half-way with soxhlet extracted XAD-2 resin which had been stored under refrigeration in methanol.

A total of 7 samples were run. Tubes 1 through 3 were control blanks (no fulvic acid), tubes 5 and 6 were Singletary Lake fulvic acid (SL104), and tubes 8 and 9 were Aldrich fulvic acid. The same amounts of fulvic acid, DEHP, and dilution water were used as in the sep-pak experiment (section 4.2.3). Tetrahydrofuran (THF) was used in place of methanol, followed by a hexane elution in place of butanol. Experimentation with different organic solvents had shown this combination to be excellent for eluting DEHP off XAD-2 resin. For completeness, a hexane wash of the sample tube was added and

the wash fraction was then counted; along with the water fraction, the THF fraction and the XAD-2 hexane fraction.

4.2.7 Phthalate Quantification of Singletary Lake Fulvic Acid (SL104)

As a control experiment, to determine whether or not any endogenous phthalate was bound to the fulvic acid (SL104), a phthalate extraction was performed.

- Four mL of $CHCl_3$: Methanol (2:1) was added to 2.5 mg of fulvic acid. Twenty five ug of Di-n-octyl phthalate was added as an internal standard. This was chosen because di-n-octyl phthalate is not found in environmental samples.
- The above sample was capped and vortexed and allowed to extract for three days.
- A blank control was run in parallel to the fulvic acid sample using the same organic solvent mixture and the same internal standard.
-After three days the control and sample were centrifuged in the same tubes they were extracted. The supernatant fraction was carefully poured into another tube and blown dry under N_2 at 60° C.
- Fifty uL of Methylene Chloride was added to the residue of both tubes, sample and control, and vortexed.
- A GC (Gas Chromatograph) chromatogram was obtained from both extracts using approximately 3 uL for injection.
- The GC program used a phthalate separation procedure (ref.6).

  Column: 1 meter x 1/8" SS, 10% OV-3 on Gas Chrom Z.

  Temperature Program: 140-230 at 8° /min, Hold at 230 .

Gas: He, 35 mL/min.

Dimethyl to di-n-octyl phthalate approximately 23 min.

4.2.8 Extractability of DEHP form Fulvic Acid SL104

To assure the validity of the previously run extraction, ^{14}C labeled DEHP was added to fulvic acid (SL104),allowed to exchange, and then extracted by the same procedure as in section 4.2.7. If all the ^{14}C labeled DEHP cold be recovered than the results of the previous experiment would be presumed valid.

- Five uL of DEHP in hexane (2.7 umol DEHP) was added to duplicate 1 mg fulvic acid samples. The solvent was evaporated under a gentle stream of N_2.
- The residue was dissolved in approximately 1 mL of water, sonicated, and then freeze dried.
- An extraction using $CHCl_3$: Methanol was done as in section 4.2.7. (No internal standard was added.)
- After 2 days the supernatant was removed and counted by adding 15 mL of Atomlight and using a liquid Scintillation counter as previously done in the binding experiments.
- The remaining residue was dissolved in water and also counted.

4.2.9 ^{14}C Diethyl hexyl phthalate (DEHP) Purity

To test the purity of the labeled DEHP used in the previous experiments, thin layer chromatography (TLC) was incorporated to separate pure from impure fractions.

- A TLC plate, soft silica gel (Analtek), was spotted with labeled and un-labeled DEHP side by side.
- The plate was run in a solvent mixture of hexane: diethyl ether:

acetic acid (120:30:1).

- The response factors of labeled and unlabeled DEHP were compared under shortwave UV. The pure DEHP spot was easily identified.
- The ^{14}C labeled DEHP spot was circled with pencil, and the silica gel above, below, and on the spot was removed by scraping with a small metal spatula, and placed respectively in three separate scintillation vials.
- Atomlight was added to each of these vials and used as an extraction solvent as well as for liquid scintillation counting.

4.2.10 Binding of ^{14}C Labeled Dioxin (2,3,7,8-TCDD) to Fulvic Acid

As done previously with phthalates an experiment was run to test the binding affinity of 2,3,7,8-TCDD to fulvic acid.

- To three glass test tubes I added 0.4 mg, 0.7 mg, and 1.0 mg of dry fulvic acid, respectively.
- Each tube received 0.5 ml of TCDD in acetone. (1,000 DPMs represented 1 ng of TCDD. Total TCDD added to each tube was approximately 4 ng.) The acetone was allowed to evaporate under a fume hood, overnight.
- Following acetone evaporation, 3 ml of water was added, the tubes were sonicated for five minutes, and gently shaken overnight.
- Two tubes containing no fulvic acid were run as blank controls.
- As with DEHP, hand packed pasteur pipettes, filled with soxhlet extracted XAD-2 resin were used for elution. The elution fractions were water, and an organic fraction consisting of 2 mL of acetone followed by 2 mL of toluene. The reaction tubes were rinsed with toluene and the XAD-2 resin was dumped into a scintillation vial

and extracted with Atomlight. Before elution each tube was pH'd, and centrifuged as done with DEHP.

- Liquid scintillation counting was done exactly the same as previous experiments. The fractions counted were:
  - water elution
  - organic elution
  - toluene rinse
  - XAD-2 resin

4.2.11 Binding of Non-Polar Organics to Clay (Kaolinite)

To test if transport of non-polar organics could be made possible by clay , rather than humics, slightly impure Kaolinite clay was obtained from Cary, North Carolina and used to test the binding affinity of 2,3,7,8-TCDD and DEHP.

A) DEHP

- 13.9, 4.7, and 4.2 mg of Kaolinite was added to three glass test tubes, respectively.
- Each tube than received 8 uL DEHP in hexane. (Refer to section 4.2.3 for DEHP concentration.)
- The hexane was evaporated under a gentle stream of N_2 and 3 mL water was added to each tube.
- Two blank tubes were prepared without the addition of Clay.
- All tubes were capped, vortexed, and shaken gently for 24 hours.
- The clay solution was then centrifuged at 3,000 rpm for 15 minutes and the supernatant was poured into respective scintillation vials.
- The clay was washed from each tube with one water rinse and each tube was then rinsed with hexane.

- The three fractions counted for each of the five tubes were:
  - supernatant
  - clay
  - hexane wash
- Liquid scintillation counting was done as in previous experiments.

B) TCDD

- 13.2mg and 9.7 mg of Kaolinite was added to two glass test tubes, respectively.
- This was followed by 0.5 mL of 2,3,7,8-TCDD in acetone. (Refer to section 4.2.10 for TCDD concentration.)
- The acetone was blown dry, under a fume hood, using a gentle stream of N_2 .
- Two tubes containing no clay were run as blank controls.
- The remainder of the procedure was the same as with the DEHP experiment.

4.2.12 Binding of Prometone and Ametryne to Fulvic Acid

A literature search revealed that some compounds were able to bind to fulvic acid by an ion-exchange mechanism (ref. 3). Two compounds were chosen from this reference to test their binding affinity for fulvic acid. The compounds chosen were Prometone and Ametryne.

Ametryne Solution; 12.2 mg dissolved in 100 ml de-ionized/distilled water (M.W. 191.30, 6.38 x 10-4 M). Diluted 20:1 (30.4 nmol/mL).

Prometone Solution: 9.0 mg dissolved in 75 mL de-ionized/distilled water (M.W. 189.26, 6.34 x 10-4 M). Diluted 20:1 (31.5 nmol/mL).

- Each solution was pH'd to neutral with 0.01M $NaCO_3$ and scanned using a UV/VIS spectrophotometer. Respective UV peaks were identified and extinction coefficients were calculated.

- Binding experiments similar to those done with DEHP and TCDD were run using fulvic acid. Detection of the bound organic was performed using UV/VIS spectraphotometry, attempting to quantitate the UV peaks identified above.

-Three mL of the diluted Prometone solution was added to each of 8 glass test tubes, respectively.

- Three tubes (nos. 1-3) received 100 uL of fulvic acid (SL104) in water (25.7 nmol).

- Three tubes (nos. 4-6) received 100 uL of Aldrich fulvic acid in water (25.3 nmol).

-Two tubes (nos. 7,8) were blank controls containing no fulvic acid.

- All eight tubes were gently shaken for 24 hours, pH'd to neutral with 0.01 M $NaCO_3$, centrifuged, and eluted through separate soxhlet extracted XAD-2 resin columns.

-The water extract was scanned for each solution from 350 nm to 200 nm against a fulvic acid reference solution of the same concentration.

- A similar experiment was run using Ametryne instead of Prometone.

4.2.13 Binding of ^{14}C Labeled DDT to Fulvic Acid

This experiment was run to give a rough estimation of the binding affinity of DDT to fulvic acid and simply to determine if binding occurred.

- 0.5 mg, 0.7mg and 0.7 mg of dry fulvic acid was added to three glass test tubes respectively.
- Ten uL of ^{14}C DDT in benzene was added to each tube. (Approximately 30,000 DPMs DDT; ^{14}C DDT 19.1 mCi/mM.)
- After evaporation of the benzene under a gentle stream of N_2 , 3 mL of water was added, the tubes were capped and vortexed, and shaken gently for 24 hours.
- Two tubes containing no fulvic acid were run as blank controls.
- After shaking, a white precipitate was detected in the samples.
- All tubes were centrifuged at 3,000 rpm for 15 min.
- The supernatant fraction was poured into a separate tube, pH'd to neutral, and eluted through an XAD-2 resin column.
- This elution was followed by 1 mL of water and 3 mL of methanol obtaining a water and an organic fraction similar to previous experiments.
- The fractions for liquid scintillation counting were:
    - supernatant (water fraction and organic fraction)
    - precipitate
    - methanol rinse of reaction tube.

4.3 RESULTS AND DISCUSSION

4.3.1 Binding of Dimethyl Phthalate (DMP) to Fulvic Acid: Sep-Pak, Procedure I

Table 4.3.1 shows the averaged DPMs for each fraction counted, tubes 1-3 contained fulvic acid (SL104) and tubes 4-5 were blank controls.

TABLE 4.3.1 DPM RESULTS OF "BINDING OF DMP TO FULVIC ACID (SEP-PAK,PROCEDURE I)."

Tube	Sample	DPMs				Total % in MeOH
		Water Fraction	MeOH Fraction	Sep-Pak Fraction	Total	
1	fulvic acid	110	4921	14	5660	87
2	fulvic acid	150	5122	18	5930	86
3	fulvic acid	57	5383	18	6131	88
*4	blank	2335	3454	25	6246	55
5	blank	56	5278	14	6008	88

* Unusually high blank value.

Note: The tables in this report which show disintegrations per minute (DPMs) do not show standard deviations of the presented values. Unless otherwise shown, an approximate relative standard deviation of + or - 10% can be assumed.

These results show that essentially no DMP came through in the water fraction. This fraction, which contained most of the fulvic acid, should have also contained any DMP that was bound to the fulvic acid. In fact, the results indicate that DMP does not bind

to fulvic acid as reported by Matsuda and Schnitzer (ref. 2). There are however two other possibilities: 1) DMP may bind to fulvic acid and than be stripped away by a C18 sep-pak cartridge or, 2) since a small fraction of the fulvic acid was not eluting with water, and only eluting after methanol was passed through the column, it is conceivable that the DMP was bound to this smaller fraction of fulvic acid and therefore was not separated from the un-bound DMP.

Addressing the first possibility requires a specific definition of binding. Using Matsuda and Schnitzer's definition (ref.2) the DMP fraction remaining in the C18 sep-pak cartridge would be considered un-bound pollutant. Their experiment separated bound from un-bound phthalate using a hexane extraction. Theoretically a C18 sep-pak cartridge separation should be equivalent to a hexane extraction procedure. These results therefore are drastically different from Matsuda and Schnitzer who claim binding of DMP to fulvic acid to be 4 to 1 molar. One should note however that this first experiment used only aquatic fulvic acid. The sep-pak procedure differed from Matsuda and Schnitzer's hexane extraction procedure in using labeled 14C DMP and counting the DMP bound to fulvic acid. The hexane extraction procedure quantitated phthalate in the hexane extraction by GC, and assumed that the difference between the starting quantity of phthalate and the GC quantitation was the amount bound to fulvic acid. This sep-pak procedure was used by Landrum et al. (ref.5) who also claim phthalate binds to fulvic acid. Their results are questionable however, because of the large phthalate breakthrough reported.

The second possibility concerning the fulvic acid remaining in the sep-pak following the water elution will be addressed in more detail by other experimentation. It should be noted however that previous research (ref.7) has shown little evidence of being able to separate chemically different fractions of fulvic acid using reverse phase columns. This would lead one to believe that while some of the fulvic acid remaining on the column could easily contain a percentage of the bound DMP, there should still be a significant amount coming through the column in the water elution if in fact DMP binds to fulvic acid. A quick glance at table 4.3.1 comparing the DPMs in the water fraction of the sample tubes to the DPMs in the water fraction of the blank tubes (no fulvic acid) shows no significant binding of DMP to the fulvic acid dissolved in the water elution. Both of the aforementioned concerns regarding a definition of binding and fulvic acid remaining on the column, will be more completely addressed in following discussions.

4.3.2 Binding of Dimethyl Phthalate (DMP) to Fulvic Acid: Sep-Pak, Procedure II

The previous experiment indicated there was no binding of DMP to fulvic acid. The specific activity of the DMP was therefore increased so even if minimal binding occurred it would be detected. This increased concentration also helped determine whether there was any significant breakthrough of DMP on C18 Sep-pak cartridges.

The primary purpose of this experiment was to determine if there was a relationship between flow rates and the breakthrough of phthalate. Three different flow rates were used and the results are presented in table 4.3.2. There was no difference in break-

through detected with the different flow rates chosen. (Flow rates were only estimates, using a 5cc syringe and timing approximate delivery of 3 mL of solution through a C18 sep-pak.)

TABLE 4.3.2 BINDING OF DMP TO FULVIC ACID (SEP-PAK,PROCEDURE II)

Tube	Flow Rate (ml/min)	Sample	DPMs		
			Water Fraction	MeOH Fraction	Total
1	60	fulvic acid	119	26334	26453
2	60	fulvic acid	131	26597	26728
3	30	fulvic acid	143	28423	28566
4	30	fulvic acid	155	30358	30513
5	10	fulvic acid	140	27650	27790
6	10	fulvic acid	137	26727	26864
7	60	blank	160	25756	25916
8	60	blank	139	27120	27259
9	30	blank	162	28920	29082
10	30	blank	177	27680	27857
11	10	blank	197	28463	28660
12	10	blank	157	28796	28953

The results also showed that DMP breakthrough remained constant and that this breakthrough is minimal in comparison to the total DMP used. A two-tailed t-test shows the mean DPM in the water fraction from the blank is significantly higher than that from the fulvic acid samples ($t = -2.9510$, $d.f. = 10$, $p = 0.012$ (i.e. <0.05)). This is very strong evidence against binding by the criteria of Landrum et al. However, it suggests that either the presence of fulvic acid decreases the water solubility of DMP, or fulvic acid-bound DMP does stick to the column. The XAD-2 results will be used to distinguish between the two possibilities. These results also led to experimentation using DEHP in place of

DMP attempting to find a pollutant that may bind to fulvic acid.

4.3.3 Binding of Diethyl hexyl phthalate (DEHP) to Fulvic Acid: Sep-Pak Procedure

DEHP was used in place of DMP because like DMP Matsuda and Schnitzer had found DEHP to bind to fulvic acid. Table 4.3.3 presents the results of DEHP binding using a C18 sep-pak cartridge as was done in the DMP binding experiments . A highly radioactive sample was used and as with DMP an almost insignificant amount of DEHP came through the sep-pak with the water elution. There was no indication of significant amounts of DEHP binding to fulvic acid. The water fraction data would suggest that about 500 - 50 = 450 DPM or about 0.3% of the DEHP could have bound to fulvic acid. This would be only 0.03 moles per mole of fulvic acid.

TABLE 4.3.3 BINDING RESULTS OF DIETHYL HEXYL PHTHALATE TO FULVIC ACID; SEP-PAK PROCEDURE.

Tube	Sample	DPMs				
		Water Fraction	MeOH Fraction	Butanol Fraction	Total-(H2O+MeOH+Butanol) (Fraction in Tube)	Total Injected
1	blank	64	2151	2756	145029	150000
2	blank	44	672	445	148839	150000
3	blank	25	2261	2376	145338	150000
*4	fulvic acid SL104	553	33760	7432	108255	150000
5	fulvic acid SL104	174	7777	858	141191	150000
6	fulvic acid SL104	204	4201	902	144693	150000
*7	fulvic acid Aldrich	692	50199	9146	89963	150000
8	fulvic acid Aldrich	313	5548	982	143157	150000
9	fulvic acid Aldrich	462	2409	1632	145497	150000

* Samples with high DPMs in the MeOH fraction and the butanol fraction. These counts do not necessarily represent binding.

This experiment also addressed the question of phthalate that may bind to fulvic acid but be stripped away by a reverse-phase column. DEHP is highly insoluble in water and therefore most of the un-bound DEHP remained in the test tube. Any DEHP that dissolved in the water or bound to fulvic acid was transferred to the column by pipetting. In examining table 4.3.3, if the DPMs of the water elution, the methanol elution, and the butanol elution are added together and compared to the DPMs injected, an indication of possible DEHP binding without the influence of the reverse-phase column can be obtained. Upon doing this comparison one can easily see that when the blank controls are subtracted from the fulvic acid sample, except for two high values obtained from tubes 4 and 7, bound DEHP is only about 2% of the total DEHP added. If this was assumed to be an upper limit of possible DEHP binding the binding ratio of DEHP to fulvic acid would not be 4 as reported by Matsuda and Schnitzer, it in fact would be 20X lower. This however is not a true estimate of DEHP binding but is only an upper limit of DEHP that could bind to fulvic acid. The experiment suggests that DEHP binding is insignificant when one compares only the water fraction which is assumed to be bound DEHP.

4.3.4 Affinity of Fulvic Acid for C18 Sep-Pak Cartridges

Prior to this experiment detection of fulvic acid, in either the elutions or on the sep-pak cartridge, was done by color observation. This observation led to the conclusion that an unknown portion of the fulvic acid was remaining on the sep-pak after the water elution and would consequently be collected in the methanol elution. It therefore became necessary to quantitate the fulvic

acid in these two fractions.

The organic carbon of each fraction, water and methanol, was quantitated by use of a $K_2Cr_2O_7$ solution. The net absorbance of these results are presented in table 4.3.4. To compute the organic carbon percentage of each fraction the absorbance value of each fraction was added together, and the absorbance value from the fraction of interest (water or methanol) was divided by this total value. This percent organic carbon value was assumed to be percent fulvic acid. A blank control was run to assure no significant amount of carbon was being stripped from the column. A standard organic carbon sample was run to compare to the fulvic acid samples. The results in table 4.3.4 show a majority of the fulvic acid would elute with the water fraction however a substantial amount would also elute with the methanol fraction. No fulvic acid remained on the column after the methanol elution. Because the objective of these experiments was to separate completely the fulvic acid from the un-bound non-polar organic of interest, and because this was not successful using C18 sep-pak cartridges, XAD-2 resin cartridges were used in place of the sep-paks. Results of a similar experiment using these XAD-2 resin cartridges, are presented in the following section.

TABLE 4.3.4 ABSORBANCE VALUES AND RESPECTIVE PERCENTAGES OF ORGANIC CARBON IN DIFFERENT ELUTIONS (WATER AND METHANOL) OF FULVIC ACID THROUGH C18 SEP-PAK CARTRIDGES.

Tube	Fulvic Acid Source	Fraction	O.D.	* O.D.	% Organic Carbon
1	Aldrich	water	301	119	61
1R	Aldrich	water	303	117	60
2	Aldrich	MeOH	345	75	39
2R	Aldrich	MeOH	347	77	40
3	SL104	water	327	108	54
4	SL104	MeOH	312	93	46
std.	none	none	219	201	100
blank	none	none	420	0	none
water	none	none	0	N/A	none

R signifies repeat analysis
STD = Standard Organic Carbon Sample (350 ug)
* O.D. = Blank (O.D.) - Sample (O.D.)

While the results of this experiment indicate C18 sep-paks were not completely successful in separating fulvic acid from unbound pollutant, the experiments using C18 sep-pak cartridges are still of worth. One should note that reverse-phase column separation of fulvic acid probably does not separate chemically different fractions. Therefore if a non-polar organic pollutant could bind to fulvic acid one would expect a respective fraction of this pollutant to pass through the reverse-phase column with the water elution. Previous experiments had shown that this was not the case. There has been no evidence of any significant amount of pollutant bound to the fulvic acid in the water fraction.

4.3.5 Affinity of Fulvic Acid for XAD-2 Resin: Hand-Packed Cartridges

The results in table 4.3.5 show absorbance values and respective percentages for organic carbon in two elutions, water and methanol, of fulvic acid (SL104) passed through XAD-2 resin cartridges. Computing results as was done in section 4.3.4 almost all the organic carbon could be found in the water elution. Therefore fulvic acid was assumed to be eluting through XAD-2 resin cartridges with the water fraction. Aldrich fulvic acid was not used, however, similar results could be expected. Prior to elution, as with the C18 sep-pak procedure, the fulvic acid was pH'd to neutral. Fulvic acid was not expected to have any affinity for XAD-2 resin at this pH, since XAD-2 resin is used as part of the preparation procedure (ref8), for concentrating fulvic acid.

TABLE 4.3.5 ABSORBANCE VALUES AND RESPECTIVE PERCENTAGES OF ORGANIC CARBON IN DIFFERENT ELUTIONS (WATER AND METHANOL) OF FULVIC ACID THROUGH HAND-PACKED XAD-2 RESIN CARTRIDGES

Tube	Fulvic Acid Source	Fraction	O.D.	* O.D.	% Organic Carbon
1	SL104	water	262	140	100
2	SL104	MeOH	404	-2	0
blank	none	none	402	0	none
water	none	none	0	N/A	none

* O.D. = Blank (O.D.) - Sample (O.D.)

4.3.6 Binding of Diethyl hexyl Phthalate (DEHP) to Fulvic Acid: XAD-2 Resin Procedure

Section 4.3.5 showed XAD-2 resin columns could be used in place of C18 sep-pak cartridges, and would be successful at separ-

ating all the fulvic acid from the un-bound pollutant. In this section experimental conditions were kept similar to section 4.3.3, using XAD-2 resin in place of C18 sep-paks. Results are given in table 4.3.6. These results gave very little indication that any statistically significant amount of DEHP bound to fulvic acid, table 4.3.6.1. The mean and standard deviation computed for the water fraction of the blank controls, fulvic acid SL104 and Aldrich fulvic acid samples, are shown in table 4.3.6.1. Subtracting the DPMs of the blank controls from the SL104 fulvic acid samples and Aldrich fulvic acid samples showed only 0.6% and 1.2% respectively, of the DEHP remained bound to the fulvic acid. Recalling that these DPMs represent accumulated scintillation count averages suggests and even larger standard deviation then is reported in the table. This could mean that the bound DEHP may even be less and is certainly far below any previously reported binding ratio (ref. 2 and 5). In fact, one may suspect DEHP does not bind to fulvic acid. These results are supportive of previous experiments using C18 sep-pak cartridges.

2) If the DPMs of the water fraction, the THF fraction, and the XAD-2 resin fraction are added together (table 4.3.6.1) representing possible bound DEHP before XAD-2 resin elution, and the blank control samples are subtracted from the fulvic acid samples, 2-tailed t-tests show $P>0.2$ for Aldrich fulvic acid vs. water and $P>0.7$ for SL104 vs. water. Therefore there is a scientific basis for concluding no evidence of binding for SL104 fulvic acid and Aldrich fulvic acid. These type of results indicate that not only is binding of tested non-polar organics insignificant when the

sample is eluted through resin columns for separation of free and bound pollutant, but that no significant binding of phthalate to fulvic acid is occurring even before elution.

TABLE 4.3.6 RESULTS OF 14C LABELED DEHP BINDING TO FULVIC ACID; XAD-2 RESIN PROCEDURE

Tube	Fulvic Acid Sample	DPMs Water Fraction	DPMs THF Fraction	DPMs XAD-2 extraction w/Hexane	DPMs Hexane wash of Sample Tube
1	blank control	96	6903	246	149719
2	blank control	110	880	64	136063
3	blank control	47	1824	98	158600
5	SL104	1429	3949	261	146868
6	SL104	812	3260	186	158861
8	Aldrich	1839	18275	3435	92563
9	Aldrich	1577	13046	681	162475

TABLE 4.3.6.1 AVERAGES OF WATER FRACTIONS; AND WATER, THF AND XAD-2 RESIN FRACTIONS ADDED TOGETHER. DPM AVERAGES ARE SHOWN FOR EACH SAMPLE; WATER, FULVIC ACID (SL104), AND ALDRICH FULVIC ACID.

Sample	Fraction	Average DPMs (approximate std. deviation)
water	water	84 (33)
Fulvic Acid SL104	water	1121 (436)
Fulvic Acid Aldrich	water	11708 (185)
water	water + THF + XAD-2 resin	3423 (3342)
Fulvic Acid SL104	water + THF + XAD-2 resin	4949 (977)
Fulvic Acid Aldrich	water + THF + XAD-2 resin	19427 (5830)

4.3.7 Singletary Lake Fulvic Acid (SL104), Phthalate Quantitation

Gas chromatograms showing phthalate peaks for the extraction of fulvic acid SL104 and a blank control were used to construct the following tables. Tables 4.3.7.1 and 4.3.7.2 show peak heights for each of the numbered peaks from the gas chromatograms (fulvic acid and blank control respectively). Tables 4.3.7.11 and 4.3.7.12 show quantitation of the respective peak heights. Quantitation was done using the following ratio:

$$\frac{\text{numbered peak height}}{\text{x (unknown quantity)}} = \frac{\text{peak height of internal standard}}{25\text{ug}}$$

The purpose of this experiment was to determine the amount of phthalate which may be bound to fulvic acid (SL104). If endogenous phthalates were bound to the fulvic acid in question then the previous experiments indicating phthalate does not bind to fulvic acid could be questionable. A comparison of table 4.3.7.11 to table 4.3.7.12 , comparing phthalates extracted from fulvic acid to phthalates extracted from a blank control shows there was no endogenous phthalate bound to fulvic acid and further supports the evidence that phthalate does not bind to fulvic acid. The observed peaks represent inevitable background from organic solvents in the laboratory. Since the other "peaks" are unidentified, but are not known phthalates, there is no point in including them.

4.3.8 Extractability of DEHP from Fulvic Acid (SL104)

This experiment was performed to test if phthalates could be

TABLE 4.3.7.1 PEAK HEIGHTS OF ORGANICS EXTRACTED FROM 2.5 MG OF FULVIC ACID (SL104). INTERNAL STANDARD, 25 ug OF DI-N-OCTYL PHTHALATE.

Peak Number	Phthalate	Peak Height (cm)
1	di-butyl phthalate	0.1
2		0.45
3		0.9
4		1.05
5		0.6
6		0.35
7	DEHP	0.1
8	di-n-octyl phthalate	4.4

TABLE 4.3.7.2 PEAK HEIGHTS FROM GC OF ORGANICS EXTRACTED FROM A BLANK CONTROL. INTERNAL STANDARD, 25 uG OF DI-N-OCTYL PHTHALATE.

Peak Number	Phthalate	Peak Height (cm)
1	di-butyl phthalate	0.2
2		0.6
3		1.6
4		1.8
5		1.2
6		0.65
7	DEHP	0.3
8	di-n-octyl phthalate	9.4

TABLE 4.3.7.1.1 QUANTITATION OF ORGANICS EXTRACTED FROM 25 MG OF FULVIC ACID (SL104). INTERNAL STANDARD, 25 ug OF DI-N-OCTYL-PHTHALATE.

Peak Number	Phthalate	Quantity (ug)
1	di-butyl phthalate	0.56
2		2.6
3		5.1
4		5.9
5		3.4
6		2
7	DEHP	0.56
8	di-n-octyl phthalate	25

TABLE 4.3.7.1.2 QUANTITATION OF ORGANICS EXTRACTED FROM A BLANK CONTROL. INTERNAL STANDARD,25 ug OF DI-N-OCTYL PHTHALATE.

Peak Number	Phthalate	Quantity (ug)
1	di-butyl phthalate	0.53
2		1.6
3		4.3
4		4.8
5		3.2
6		1.7
7	DEHP	0.8
8	di-n-octyl phthalate	25

extracted from fulvic acid by the method used in section 4.3.7 ^{14}C labeled DEHP was given the chance to bind to fulvic acid or to exchange with unlabeled DEHP already bound to fulvic acid. Results in table 4.3.8 show excellent extractibility of the ^{14}C labeled DEHP. From these results one could assume any phthalate bound to fulvic acid (SL104) would be extracted using the chloroform/methanol procedure.

TABLE 4.3.8 RESULTS OF EXTRACTABILITY OF DEHP FROM FULVIC ACID (SL104)

	DPMs		
Tube	$CHCl_3$:CH_3OH Extraction	Water Fraction (non-extracted DEHP)	% Extracted
1	128167	4880	96
2	143949	2346	98
Avg.	136058	3613	97

4.3.9 ^{14}C Labeled Diethyl hexyl Phthalate (DEHP) Purity

DEHP like any compound becomes more impure with time. This experiment was performed to test the purity of the DEHP being used. TLC was used to separate pure DEHP from impure metabolites. Unlabeled DEHP was spotted alongside labeled DEHP from which the response factor of pure DEHP was determined. Once the pure fraction of labeled DEHP was identified and separated from impure fractions, which were above and below the DEHP spot, all fractions were counted and the DPMs of each fraction was recorded. The experiment was run twice and results are presented in table 4.3.9. This table shows the ^{14}C labeled DEHP in these experiments to be 97.5% pure.

TABLE 4.3.9 RESULTS OF THE DEHP PURITY EXPERIMENT USING THIN LAYER CHROMATOGRAPHY (TLC) AND 14C LABELED DEHP

Vial No.	Position on TLC Plate	DPMs	% of Compound
1	below spot	524	0.9
2	spot (pure DEHP)	58617	97.5
3	above spot	993	1.7
4	below spot	503	0.9
5	spot (pure DEHP)	56426	97.4
6	above spot	1077	1.8

4.3.10 Binding of ^{14}C Labeled Dioxin (2,3,7,8,-TCDD) to Fulvic Acid (SL104)

Due to the increasing interest in dioxin and its' fate in the environment similar binding experiments were done using ^{14}C labeled TCDD in place of DEHP or DMP. However, since dioxin is extremely insoluble in water it was given a chance to bind directly to fulvic acid without the use of a water phase. This was done by injecting TCDD in acetone, directly on dry fulvic acid and then allowing the acetone to evaporate. The fulvic acid was then dissolved in water and eluted through XAD-2 resin. Because of the extreme toxicity of dioxin much smaller quantities of dioxin were used when compared to quantities of phthalates used in previous experiments. Results are presented in table 4.3.10.

TABLE 4.3.10 RESULTS OF 2,3,7,8 - TCDD BINDING TO FULVIC ACID (SL104).

Tube	Quantity of fulvic acid(mg)	DPMs Water Fraction	DPMs Organic Fraction	Acetone Wash of Sample Tube	XAD-2 Resin Fraction	Total
1	0.4	1263	1787	759	81	3890
2	1	1120	1548	1063	92	3823
3	0.7	850	1849	1061	100	3860
4	blank control	1535	1651	516	82	3784
5	blank control	571	1627	1647	106	3951

As with phthalate, dioxin appeared to have no significant binding affinity for fulvic acid. The water elutions for fulvic acid show no larger quantities of TCDD than do the water elutions for the blank controls. Because XAD-2 resin was used in place of C18 sep-paks, no fulvic acid remained on the column.

The organic solvent elution of acetone and toluene showed no lesser amount of TCDD when fulvic acid was present. These results indicate TCDD does not bind to aquatic fulvic acid and began to cast doubt on the general perception that non-polar organic pollutants are transported through the environment by binding to fulvic acid. Perhaps transport is accomplished through another medium (eg. clay) and this became the focus of the next series of experiments.

4.3.11 Binding of Non-Polar Organics to Clay (Kaolnite)

Concluding it was very unlikely that non-polar organics like phthalates or even dioxin were transported through the environment by fulvic acid a simple experiment was devised to test the affinity of these compounds for clay. The intent of these experiments was not to quantitate non-polar organic binding to clay but to determine if it might be possible, thereby postulating a method of transport.

The clay concentrations chosen were representative of actual environmental conditions. This same reasoning had been used for fulvic acid concentrations; however while concentrations used for fulvic acid represented possible environmental conditions they were generally high for most any lake or body of water where fulvic (humic) acid would be found. This was done for ease in experimentation. The clay concentrations however were not at all unreasonable for many lakes and streams.

Clay is not soluble so resin cartridges were not used. Instead after a given time for equilibrium the samples were centrifuged and two fractions counted, the supernatant and the clay precipitate. Experimental conditions were kept as close as possible to previous experiments rinsing the tubes with the appropriate organic solvent after each clay extraction. Results of ^{14}C labeled TCDD are shown in table 4.3.11.2 and those for ^{14}C DEHP are shown in table 4.3.11.1. Both experiments give an indication that these non-polar organics may bind to clay. Blank controls were ran precisely the same as the experimental samples. While the experiment was intended only as an approximation of actual conditions, it gave evidence of non-polar organic pollutants binding

TABLE 4.3.11.1 BINDING OF NON-POLAR ORGANICS TO CLAY (KAOLINITE) 14C DEHP RESULTS

Tube	Quantity of Clay (mg)	DPMs Water Fraction	Clay Fraction	Hexane Wash	Total
1	13.9	32982	112244	2858	148084
2	4.7	107125	40593	3949	151667
3	4.2	96576	53966	2983	153525
4	blank control	143948	*1603	5847	151398
5	blank control	152949	622	276	153847

* Clay fraction for the blank controls is a water rinse of tubes.

TABLE 4.3.11.2 BINDING OF NON-POLAR ORGANICS TO CLAY(KAOLINITE), 14C 2,3,7,8 - TCDD RESULTS.

Tube	Quantity of Clay (mg)	DPMs Water Fraction	Clay Fraction	Acetone Wash	Total
1	13.2	716	2665	345	3726
2	9.7	874	2630	447	3951
4	blank control	2047	857	918	3822
5	blank control	1689	1224	789	3707

to clay.

4.3.12 Binding of Prometone and Ametryne to Fulvic Acid

Previous binding theories had suggested hydrophobic interactions (ref.2) and possible hydrogen bonding (ref.2) between non-polar organics and fulvic acid. These theories were the basis for the previous experiments with DMP, DEHP and TCDD. Since none of these experiments gave an indication of significant interaction between the compounds chosen and fulvic acid (aquatic or Aldrich), the next objective was to explore other possible compounds binding by a different mechanism. The reason for doing this was to test the experimental method chosen, and assure it was a reasonable method for determining binding interactions.

A research paper by Senesi and Testini (ref.3) suggests electron donor-acceptor processes involved in herbicide - humic acid interactions. Two compounds, Ametryne and Prometone, were chosen from this paper to test their binding properties to aquatic and Aldrich fulvic acid. The experiment was ran precisely as the DEHP and TCDD experiments using hand - packed XAD-2 resin columns with only one minor change. Neither the Ametryne nor Prometone were radio-labeled and a new method of detection had to be devised, therefore UV absorption was chosen.

Both compounds were scanned from 350mn to 200nm, and a sharp UV peak was found for each compound. (219nm for Prometone and 223nm for Ametryne.) If the compound were to bind to fulvic acid, and if the UV peak didn't shift when bound to fulvic acid, quantitation of percent herbicide bound could be achieved if bound and free herbicide could be separated. Hence the use of XAD-2 resin

columns which had been used in previous experiments. There were problems however, with results for Prometone presented in table 4.3.12.

TABLE 4.3.12 RESULTS OF THE PROMETONE BINDING EXPERIMENT

Tube	fulvic acid sample	Absorbance of Water Elution at 219nm
1	SL104	0.239
2	SL104	0.44
3	SL104	*0.087
	Average	0.340 + or - 0.140
4	Aldrich	0.262
5	Aldrich	0.383
6	Aldrich	0.429
	Average	0.358 + or - 0.086
7	blank control	0.378
8	blank control	0.311
	Average	0.345 + or - 0.047

* Value not included in average

These results suggest Prometone does not bind to fulvic acid when one compares absorbance values of samples against absorbance values of blank controls. However, problems encountered in this particular procedure were different from previous experiments and were much more difficult to overcome. These problems led the researchers to believe that no conclusive evidence of binding or non-binding could be obtained using these procedures.

The first problem was breakthrough of free herbicide. The amount of breakthrough was large and varied for each column. It was difficult to obtain a meaningful average for blank controls that could be subtracted from fulvic acid samples especially since

this breakthrough represented such a large portion of total herbicide eluted. Phthalates and dioxin had not presented a problem of significant breakthrough on XAD-2 resin.

The second problem was the appearance of a new peak with the fulvic acid samples. A fulvic acid solution of similar concentration as the samples, was eluted through XAD-2 resin without the herbicide of interest. This was used as a reference for the UV spectrophotometer when scanning fulvic acid - herbicide samples also eluted through XAD-2 resin columns. The use of fulvic acid solution as a reference solution was to eliminate the rather complex UV absorption spectrum of fulvic acid. However, in the case of the herbicides in question, a new peak appeared in the spectrum. It was suspected that this new peak represented herbicide - fulvic acid interaction however this could not be proven and the peak could not be quantitated. Without radio-labeled herbicide, conclusive evidence of binding or non-binding could not be shown. This experiment involving herbicides did not resolve questions concerning the experimental method used to separate free from bound species since evidence of herbicide binding could not be proven.

4.3.13. Binding of ^{14}C Labeled DDT to Fulvic Acid

DDT was believed to be another pollutant capable of binding to fulvic acid (ref.4). The experimental objective was to show DDT would bind so that the experimental method could be proven valid. ^{14}C labeled DDT was obtained and the same type of experiment was conducted. There was an unusual result however when DDT was added to the tubes containing fulvic acid. Upon addition of the water

and vortexing to dissolve the fulvic acid a white fibrous precipitate began to settle on the bottom of the tube. The tubes were allowed to shake for 24 hours and after shaking were processed in the usual manor with the exception of the white precipitate which was spun down and separated from the supernatant after which it was counted as a separate fraction. The results of these counts are given in table 4.3.13.

TABLE 4.3.13 RESULTS OF DDT BINDING EXPERIMENT

		DPMs				
Tube	Sample	Water Fraction	Organic Solvent Fraction	Precipitate Fraction	MeOH Wash	Total
1	fulvic acid SL104	386	1434	24279	1949	28048
2	fulvic acid SL104	681	1412	24276	2682	29051
	fulvic acid SL104	623	1735	26910	762	30030
4	blank control	83	1414		25889	27386
5	blank control	147	1049		27550	28746

It seems probable that the DDT and fulvic acid formed a complex which became almost insoluble in water or at least in the quantity of water used for this experiment (3mL.). As seen from table 4.3.13, most of the ^{14}C labeled DDT was found in the precipitate. The supernatant lost its color indicating fulvic acid was no longer dissolved and was believed to be part of the precipitate. In the blank controls most of the DDT was found left in the tube. It also appeared as if a small portion of the DDT - fulvic acid complex was soluble in water and passed through the XAD-2 resin column as predicted. The table shows 5X the DPMs found in

the water fraction for the fulvic acid solution than in the water fraction for the blank control. No significant difference was seen in the organic solvent elution between the samples and controls and this may be attributed to a small fraction of DDT that remained unbound but found its way into the column. The experiment had unexpected results but indicated DDT binds to fulvic acid and while it wasn't strong evidence the experiment gave reason to believe the method used for separating bound from un-bound pollutant may be valid.

4.4 CONCLUSIONS

The original objective of this experiment was to determine binding affinities of chosen non-polar organic pollutants to fulvic acid. DMP, DEHP and 2,3,7,8 TCDD were not found to bind to either aquatic or Aldrich fulvic acid. While the evidence gathered is not conclusive it strongly suggests that in fact these compounds do not bind to fulvic acid. This evidence conflicts with previous findings by other researchers and suggests new mechanisms of pollutant transport in the environment.

These experiments also proved C18 sep-pak cartridges to be in-effective for separating pollutant bound to fulvic acid from unbound pollutant. This conflicts with previous findings of Landrum et al. (ref.5). Instead hand packed XAD-2 resin cartridges were found to be useful, however, as in the case of Ametryne and Prometone not all pollutants can be separated from fulvic acid using these resin columns. XAD-2 resin is effective for fulvic acid elution, providing the solution has been previously neutralized.

XAD-2 resin became the method of choice after successive experimentation.

Non-polar organics, DEHP and 2,3,7,8-TCDD were able to bind to clay. This experimentation suggests another method for pollutant transport in aquatic environments and certainly provides new possibilities of further research in the area of non-polar pollutant binding. While only two experiments were performed, and very little information was actually gathered, and no attempt was made to quantitate amount of pollutant bound, the evidence for significant binding of non-polar organics to clay was much greater than evidence of non-polar organic pollutants binding to fulvic acid.

DDT was the only pollutant of those tested that appeared to bind strongly to fulvic acid. No attempt was made to determine the binding mechanism. Mechanisms of binding for non-polar organics had previously been postulated as either hydrophobic interactions or H-bonds but this appeared not to be the case in the experiments performed since binding of phthalate or dioxin could not be shown. Ionic inter-actions are believed to be important in herbicides like Ametryne and Prometone but results for these compounds were inconclusive. The fulvic acid - DDT interaction was also unusual in that it formed a precipitate that may indeed be water soluble if larger quantities of water are used. The DDT - fulvic acid interaction may be of interest for further research in the area of pollutant - fulvic acid binding since there appears to be strong bonds between these two compounds.

REFERENCES

1) G. Ogner and M. Schnitzer, Science, 170, 317-318 (1970).

2) K. Matsuda and M. Schnitzer, Bull. Environ. Contam. Toxicol., 6, 200-204 (1971).

3) N. Senesi and C. Testini, Cheomsphere, 13, 461-468 (1984).

4) C. W. Carter and I.H. Suffet, Environ. Sci. Technol., 16, 735-740 (1982).

5) P.F. Landrum , S.R. Nihart, B.J. Eadie, and W.S. Gardner, Environ. Sci. Technol., 18, 187-192 (1984).

6) P.W. Albro, R. Thomas, and L. Fishbein, J. Chromatogr., 76, 321-330 (1973).

7) E. T. Gjessing, Physical and Chemical Characteristics of Aquatic Humus, Ann Arbor Science Pub., (1976) pp. 15-85.

8) E. M. Thurman and R. L. Malcom, Environ. Sci. Technol., 15, 463-466 (1981).

9) J.S. Amenta, J. Lipid Res., 5, 270-272 (1964).

SUMMARY

Presented in this report were four separate sections of experimental procedures and results along with a discussion and conclusion for each section. Section one presented results for preliminary experiments. From the various experiments performed I was able to learn more about the structure of fulvic acid and possible directions from which my research could branch.

Once a successful methylation of fulvic acid was achieved I developed a new method to determine carboxyl content. This was done by use of a radioassay procedure. An advantage of this procedure over previous carboxyl determination procedures was being able to use a small quantity of material to accurately determine total carboxyl content. This section was subsequently submitted for publication.

In section two I presented various methods for functional group determination. The objective was to better characterize the fulvic acid of interest. (Aquatic fulvic acid obtained from Lake Singletary North Carolina.) These results can be compared to previous reports which show functional group content for fulvic acid obtained from other sources. Of particular interest are the lack of phenolic hydroxyls found in Lake Singletary fulvic acid.

Section four was done as a follow-up to sections one, two and three. The original objective was to present methods which could easily characterize any fulvic acid using only a small fraction of sample. One way this can be done is by determining binding properties associated with the particular material. Other reports

have shown fulvic acid to be able to bind to a variety of different organic pollutants. I was interested in the binding mechanism. Several organic pollutants were tried. Except for DDT I found no conclusive evidence, under conditions of pH that can actually occur in natural waters, supporting previous claims of binding. In fact the experimental evidence presented in section four suggests that fulvic acid does <u>not</u> bind to various non-polar organic pollutants.