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CHROMATOGRAPHIC AND SPECTROSCOPIC STUDIES ON AQUATIC FULVIC ACID

DISSERTATION

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High Performance Liquid Chromatography (HPLC) was used to investigate the utility of this technique for the analytical and preparative separation of components of aquatic fulvic acids (FA). Three modes of HPLC namely adsorption, anion exchange and reversed phase were evaluated. Aquatic fulvic acids were either extracted from surface water and sediment samples collected from the Southwest of the U.S., or were provided in a high purity form from the USGS.

On the adsorption mode, a major fraction of aquatic fulvic acid was isolated on a semipreparative scale and subjected to Carbon-13 NMR and FAB Mass Spectroscopy. Results indicated that (1) The analyzed fraction of fulvic acid contains more aliphatic than aromatic moieties; (2) Methoxy, carboxylic acids, and esters are well-defined moieties of the macromolecule; (3) Phenolic components of the macromolecules were not detected in the Carbon-13 NMR spectrum possibly because of the presence of stable free radicals.

Results of the anion exchange mode have shown that at least three types of acidic functionalities in aquatic fulvic acid can be separated. Results also indicated that aquatic fulvic acid can be progressively fractionated by using subsequent modes of HPLC.

Results of reversed phase mode have shown that (1) The fractionation of aquatic fulvic acid by RP-HPLC is essentially controlled by the polarity and/or pH of the carrier solvent system; (2) Under different RP-HPLC conditions aquatic fulvic acid from several locations are fractionated into the same major components; (3) Fulvic acid extracted from water and sediment from the same site are more similar than those extracted from different sites; (4) Cationic and anionic ion pair reagents indicated the presence of amphoteric compounds within the polymeric structure of fulvic acid.

Each mode of HPLC provided a characteristic profile of fulvic acid. The results of this research provided basic information on the behavior of aquatic fulvic acids under three modes of HPLC. Such informations are prerequisite for further investigation by spectroscopic methods.

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CHAPTER I

INTRODUCTION

Organic matter in the aquatic environment originates from natural as well as synthetic sources. The natural organic fraction is formed in association with normal activities of the biological community. This fraction is composed of various compounds such as proteins, carbohydrates, amino acids, humic and fulvic acids. The synthetic fraction is a result of man's activities during the past decades. Examples of these compounds are organic pesticides, polychlorinated biphenyls (PCB's), polynuclear aromatics (PNA's), and phthalate esters (PE's).

Humic substances (HS) constitute the major component of aquatic organics which are composed of amorphous, brown or black, hydrophilic, acidic, polydisperse substances. Based on their solubility in alkali and acid, humic substances are usually divided into three main fractions: (a) Humic acid (HA) which is soluble in dilute alkaline solution but is precipitated by acidification of the alkaline extract, (b) Fulvic acid (FA) which is soluble in both acid and base ; and (c) Humin which is the humic fraction that cannot be extracted by dilute base and acid (1). There is increasing evidence that the chemical structure and properties of the humin fraction are similar to those of humic acid, and that

its insolubility arises from the firmness with which it combines with inorganic soil and water constituents. Data available at this time suggest that structurally the three humic fractions are similar to each other, but that they differ in molecular weight, ultimate analysis, and functional group content. It also suggests that the fulvic acid fraction has a lower molecular weight, containing more oxygen but less carbon and nitrogen, and having a higher content of oxygen-containing functional groups (CO_2H, OH, C=O) per unit weight than humic acid and the humin fraction. Figure 1 shows a typical fractionation scheme for Humic substances in soils and sediments.

Humic compounds lack the specific chemical and physical characteristics such as melting point, refractive index and exact elementary composition, usually associated with well defined organic compounds. Important characteristics exhibited by all humic fractions are resistance to microbial degradation, and ability to form stable water-soluble and water-insoluble salts and complexes with metal ions and hydrous oxides and to interact with clay minerals and organic chemicals often added by man, which may be toxic pollutants.

Fulvic acid, the acid and base soluble fraction of aquatic humus, is one of the most abundant naturally occurring organic compounds that may constitute up to 80% of

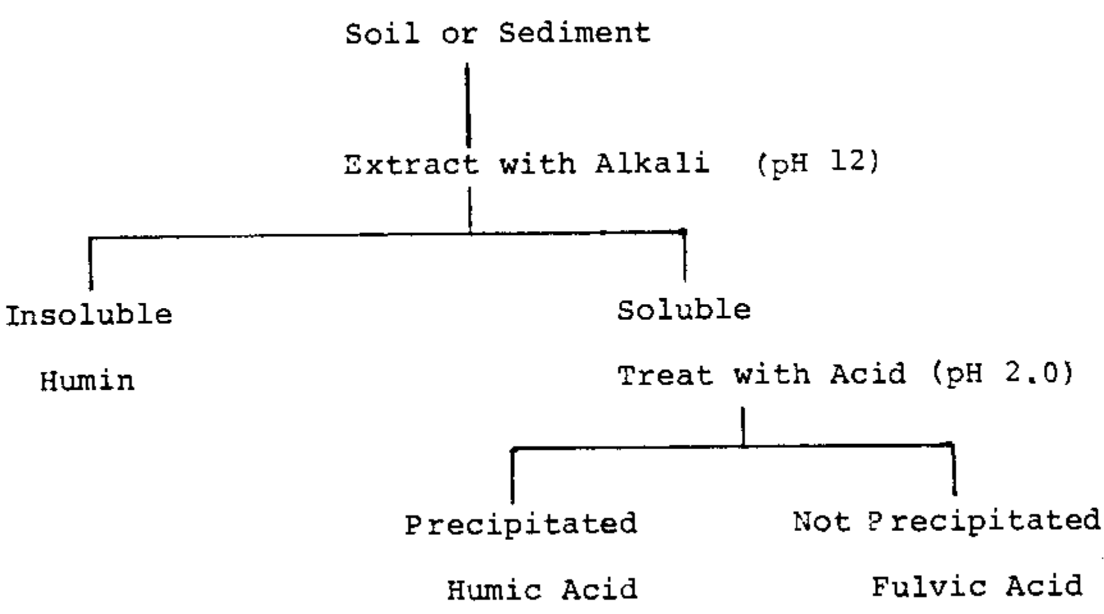


Figure 1--Fractionation of humic substances (from ref. 1).

aquatic humus and is known to be chemically and biologically stable. Fulvic acid plays an important role in many environmental reactions such as complexation with heavy metals (2) formation of trihalomethanes upon chlorination (3) and solubilization of organic pesticides (1). The exact structure of fulvic acid is unknown. However, it is known to be a polymeric material of molecular weight ranging from few hundreds to several thousands. Elemental composition of a typical aquatic fulvic acid is 50% C, 40% O, 3-4% H and 1-2% N. The macromolecules are known to have several oxygen functional groups such as carboxyl, keto, phenolic and methoxy groups (4). Figure 2 shows one of the early proposed structures of fulvic acid. Figure 3 shows some of the recently proposed structural components of fulvic acid.

Current State of Knowledge

Fulvic acid has been extensively investigated by chemical and spectroscopic methods in the past few decades. The preponderance of evidence indicates that fulvic acid behaves like linear flexible polyelectrolytes that are readily aggregated at low pH with the aid of hydrogen bonding, Van-der Waals interactions and interactions between the π electron system of adjacent molecules (4). As the pH increase, these forces become weaker, and because of increasing ionization of CO_2H and phenolic OH groups, particles separate and begin to repel each other

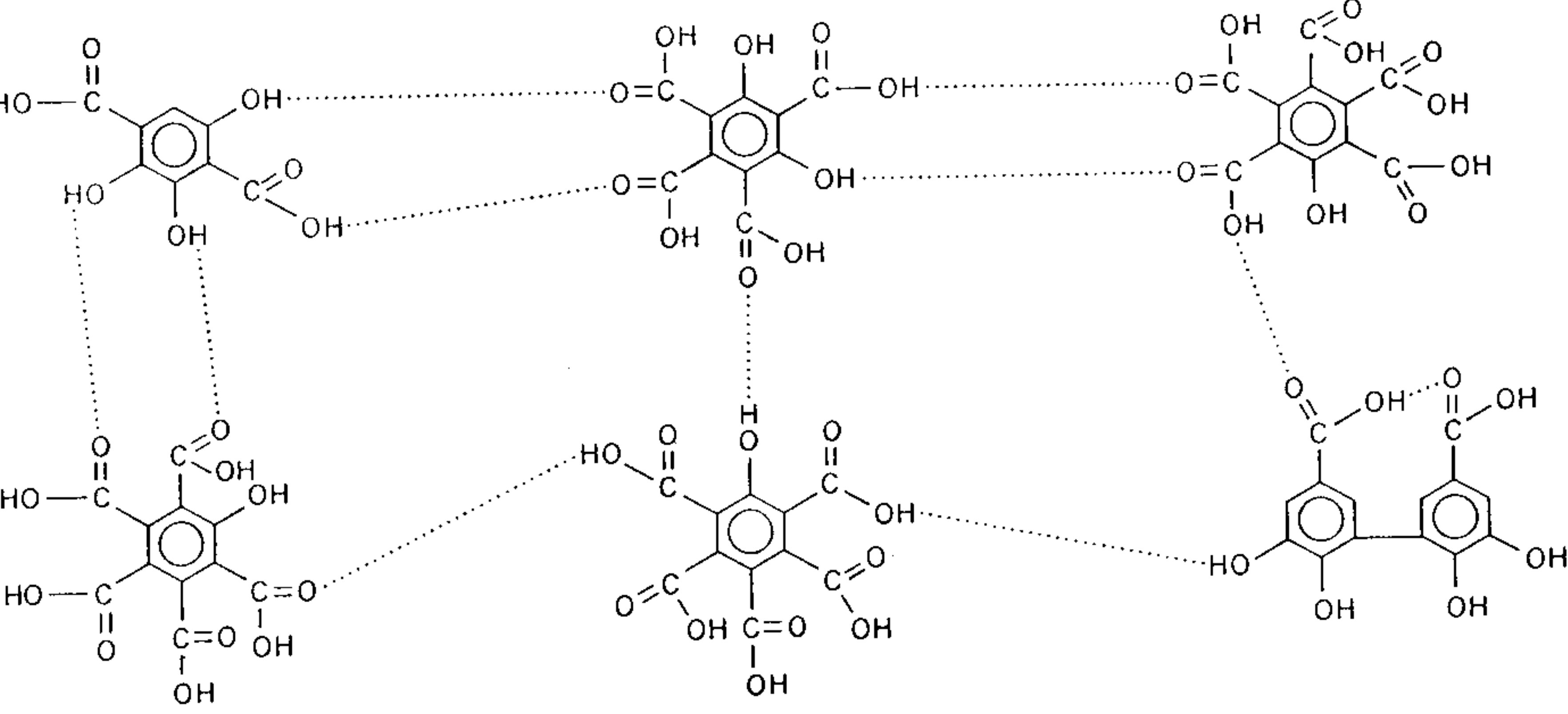


Figure 2--Proposed structure of fulvic acid (from ref. 1).

TERPENOIDS (AGATHIC ACID)

STEROID

LIGNIN FRAGMENT

HYDROLYZABLE TANNINS

FLAVONOIDS

Figure 3--Possible structural components in fulvic acid from Suwannee River (from ref. 11).

electrostatically, so that the molecular arrangements become smaller and smaller but better oriented (5). At pH values between 2-3, fulvic acid occurs as elongated fibers and bundles of fibers with a relatively open structure. With increasing pH, the fibers tend to mesh into a woven network yielding a sponge-like structure (5). The carbon skeleton of fulvic acid consists a broken network of poorly condensed aromatic rings with an appreciable number of disordered aliphatic chains and alicyclic structures (4, 6).

Degradation Studies

Because of the chemical complexity of humic materials, many workers (7, 8, 9) have used degradative methods, hoping to produce compounds that could be identified and whose structures could be related to those of the starting materials. At times the methods were too mild to yield products that were identifiable; on other occasions the methods were so drastic that they produced only oxalic acid and acetic acids in addition to CO_2 and H_2O, none of which provided useful structural information. The degradative methods that have been used on humic substances are of three types: oxidative, reductive, and biological. In general, oxidative degradation has been more successful than reductive degradation. This is so because humic substances contain considerable amounts of oxygen and are difficult to reduce.

Oxidative degradation of humic substances traditionally involves harsh oxidative conditions using alkaline permanganate and copper oxide (5, 1, 10). The products of these conditions are monocarboxylic acids, dicarboxylic acids and benzene carboxylic acids. These products are the skeletal remains of oxidation, because the powerful oxidative agents take one-third of the molecules to form carbon dioxide (10). These products have given us an idea of the structural core of humic substances - that is, an aromatic core with aliphatic chains (11). Milder oxidative conditions, such as alkaline hydrolysis with 5N NaOH at 170 ^{0}C or KOH fusion, have been tried (1, 10). Phenols and phenolic acids were found as well as aromatic and aliphatic acids. These data suggest a core of aromatic content with ester bridges of aliphatic content (10).

Reductive procedures, such as zinc dust distillation and Na amalgam reduction, have not been productive, because of the large oxygen content of the humic material; yields are only 1% or less (1). Schnitzer and De Serra (12) concluded that compared to alkaline $KMnO_4$ oxidation of methylated HA's and FA's, Na-amalgam reduction is relatively inefficient method that tells little about the chemical structure of humic materials.

Recent chemical degradation followed by GC-MS or proton and Carbon-13 NMR studies have established the presence of

aliphatic structures in addition to the phenolic moieties in fulvic acid (13-21).

Chromatographic Studies

Until very recently, conventional gas chromatographic methods have had serious limitations in investigations of naturally occurring organics in aquatic systems due to their non-volatile nature. By contrast, High Performance Liquid Chromatography (HPLC) continues to be one of the most effective means for isolation and fractionation of aquatic organics. HPLC is ideally suited to the separation of complex mixtures of organic molecules including highly polar and non-volatile compounds. Different modes of liquid chromatography were used in fulvic acid research to determine its molecular size or to purify and fractionate its components. Gel filtration using Sephadex has been utilized by some investigators (22, 23) to determine the molecular size and subsequently the molecular weight of fulvic acid. The weight average and number average molecular weights ranged from 600 to 100,000. These wide ranges were attributed to differences in methodologies, standards and sources of fulvic acid. Problems inherent in this technique are reported to be: (1) Adsorption of humic substances onto the gel (24); which retards the elution of the material and results in an underestimate of molecular weight. (2) Presence of slight negative charge on the

resin. This results in repulsion between the resin and negatively charged humic substance molecules. Thus, these molecules travel the column more quickly than a noncharged solute of similar size, and an error in molecular size is made, the molecule appears larger than its actual size (25). These problems, coupled with the fact that major size differences occur between humic acid and fulvic acid, but not among fulvic acids, limits the use of this type of chromatography (11). Size separation of fulvic acid is difficult because of the small range in molecular weight of aquatic fulvic acid (from 1000 to 2000) (5, 26).

HPLC studies using weak anion exchange resins such as diethylaminoethyl (DEAE) and pellionex (WAX) have been used to fractionate non-volatile organics from surface and chlorinated waters (27-29). Variable resolution was obtained depending on the column configuration, type of solvent and gradient conditions. These resins may also be used for chromatography of humic substances. This method has been useful for separating a phenolic-rich fraction from aquatic fulvic acids. In this type of chromatography the mechanism of separation is based on the interaction between phenolic and carboxylic groups and the nitrogen groups present on the weak anion exchange resin.

Macroreticular resins such as XAD 102, 104, 107 and 108 have been extensively used to purify and concentrate natural

organics from water samples (30-35). These resins are nonionic, macroporous copolymers which possess large surface areas. In this type of adsorption chromatography the hydrophobic part of the solute molecules binds or adsorbs to the resin. The polar groups (hydroxyl and carboxylic groups) orient into the aqueous phase. Adsorption and desorption may be controlled by balancing the polar and nonpolar interactions. Adjusting the pH to 2.0 makes the humic substances nonionic, and thus adsorb onto the resin. At pH 5.0 or greater, the solute molecules are desorbed, because of the ionic character of the carboxyl group (11). Several investigators have used the resins as packing material for HPLC columns. Elution patterns for several simple organic molecules are reported (36-37). Separate studies have reported the fractionation of fulvic acid into 2, 3 or 4 fractions using XAD-108 (chromosorb-108) with different solvents and gradient conditions (38, 39). The mobile phase includes 0.1 M Prideaux buffer (0.1 M in phosphoric, acetic and boric acids) and increased percentage of sodium hydroxide or 0.1 N phosphoric acid and 0.1 N sodium hydroxide.

Only few studies have reported the utilization of reversed-phase (RP) columns for fractionation of non-volatile organics from water (40-44). Reversed-phase HPLC, through the selection of appropriate bonded phase and

carrier solvent system, is ideally suited for separation of organic compounds of a wide range of polarity. Also through the use of paired ion reagents RP columns can be adapted for separation of ionic components. Also ion-pair chromatography (45-50) has been shown to be valuable for HPLC of ionizable compounds and to possess great versatility. This technique has been used to separate biogenic amines, sulfonamides and other pharmacologically active material (46), sulfonic acids (51), carboxylic acids and phenols (52-54). Such criteria are ideally suited for fractionation of fulvic acid components

Spectroscopic Studies

One of the major difficulties in humic material research is the limitations of spectroscopic instruments to identify the molecular structure of high molecular weight complex molecules. Recently, there has been a major breakthrough in spectroscopic instrumentation which allowed the establishment of the structures of several complex macromolecules of biochemical and medical importance (55, 56).

A number of structural studies on humic and fulvic acid have used NMR, but the spectra obtained have yielded only general information (57, 58). The proton NMR spectra showed line broadening and line shifting, apparently due to hydrogen bonding and presence of exchangeable protons, and

the Carbon-13 spectra consisted of a few broad, weak lines. Derivatization allows labeling of the hydroxyl groups and elimination of hydrogen bonding (15).

A generalized model for the chemical structure of humic acids was proposed in which humic acid molecules formed both homogeneous and heterogeneous aggregates (59). Recent studies indicate that a similar model applied to fulvic acid (15). These aggregates are held together by various weak-bonding mechanisms, the most important of which is hydrogen bonding between the carboxylic acid groups, phenolic groups, and other hydroxyl groups. The distribution and concentration of these functional groups may be determined by using Carbon-13 labeled methyl derivatives of these groups which are prepared and analyzed by Carbon-13 NMR.

There are several inherent advantages that Carbon-13 NMR spectroscopy has over Proton NMR for structural studies of humic material. First, the carbon skeleton of the humic material is observed rather than the adjacent protons, which allows functional groups such as ketones to be detected. Secondly, the carbon nuclei are spread over a wider range of chemical shifts in Carbon-13 NMR, and consequently separate signals are observed even when carbons have only small differences in structural environments. An added advantage is that line widths of signals in Carbon-13 NMR are smaller

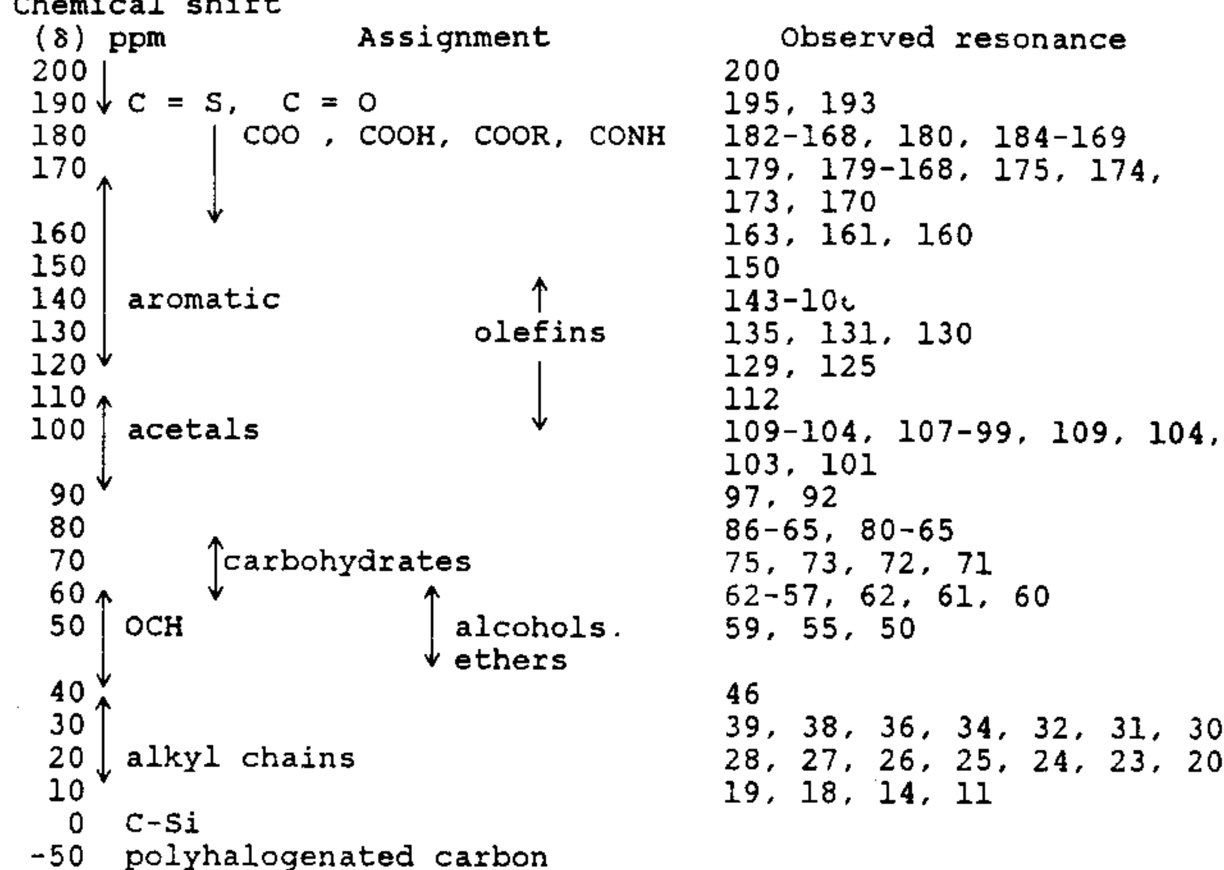
than in Proton NMR, hence if overlap of signals does occur it is less pronounced.

The Carbon-13 NMR data obtained for humic substances (62) are summarised in Table I. Several points are clear: (1) Carbon-13 NMR spectroscopy confirms the presence of polymethylene in humic substances. Carbons α, β, and γ from the end of alkyl chains have also been found. (2) Signals from alcohols and ethers have been identified. In one case it has been possible to identify primary and secondary alcohols and acetals. (3) Most spectra show signals from aromatic carbon, but in some samples which had been extensively dialysed aromatic signals were absent. (4) Signals from carboxyl carbons are nearly always observed. They originate from carboxylic acids, and possibly also from amide linkages in proteins. There is little evidence for the presence of aldehydes or ketones in humic substances.

Carbon-13 NMR spectrometry is one of the most powerful tools that provides information on the carbon skeleton of organic molecules. The recent development of cross polarization magic angle spinning (CP-MAS) technique has overcome inherent problems due to dipole-dipole interactions and length of spin-lattice relaxation times. In the CP-MAS technique protons are decoupled from Carbon-13 nuclei and then used to enhance the relaxation of the Carbon-13 nuclei which result in improved resolution and sensitivity (60-64).

TABLE I

CARBON-13 RESONANCES IN HUMIC MATERIALS (FROM REFERENCE 62)



Chemical shift (δ) ppm	Assignment	Observed resonance
200		200
190	C = S, C = O	195, 193
180	COO , COOH, COOR, CONH	182-168, 180, 184-169
170		179, 179-168, 175, 174, 173, 170
160		163, 161, 160
150		150
140	aromatic	143-10[illegible]
130	olefins	135, 131, 130
120		129, 125
110		112
100	acetals	109-104, 107-99, 109, 104, 103, 101
90		97, 92
80		86-65, 80-65
70	carbohydrates	75, 73, 72, 71
60		62-57, 62, 61, 60
50	OCH; alcohols, ethers	59, 55, 50
40		46
30		39, 38, 36, 34, 32, 31, 30
20	alkyl chains	28, 27, 26, 25, 24, 23, 20
10		19, 18, 14, 11
0	C-Si	
-50	polyhalogenated carbon	

Aromaticities and carboxylic acid content of fulvic acid were calculated from the NMR spectra and the results were compared with those previously obtained on the same samples by chemical methods (65). It was reported that chemical techniques have overestimated the degree of aromaticity and underestimated the carboxylic acid content of humic substances (16, 66).

Fast atom bombardment (FAB) mass spectroscopy has recently been developed to study organic salts, polar antibiotics, nucleoside phosphates, and underivatized peptides (67). FAB uses the sputtering of ions by fast ion bombardment to produce both positive and negative ion mass spectra with equal facility and without the need to make any changes in the atom source conditions. The sputter source eliminates the necessity to volatilize the sample prior to ionization. The ionization process should give, in abundance, ions indicative of the molecular weight of the compound, and, additionally, structurally relatable fragmentation of the molecule should be in evidence. FAB is available using chemical ionization that gives spectra on compounds with molecular weights of 500 or more (67, 68). This tool gives promise for fulvic acid fractions, especially those isolated from water, where molecular weights range from 500 to 2000. If methylation procedures are used prior to MS, this may increase volatility so that

solid probe high-resolution mass spectroscopy will be an important tool for humic structural studies (11). The technique is evolving as one of the most powerful tools to study polar high molecular weight compounds (69, 70).

Recently, FAB mass spectroscopy coupled with HPLC column has been used for separation and identification of organics isolated from river and drinking waters (71). Methanol extracts of nonvolatile organics from samples of river and drinking water were obtained by either freeze-drying or by XAD-2 resin adsorption. Separation and fractionation of water extracts was carried out by using either normal or reversed-phase HPLC. Results show complex mixtures of organics with a wide diversity of structural types have been present in water sample. Among the nonvolatile organics identified are polychlorinated terphenyls, nonionic and cationic surfactants, pharmaceuticals, peticides, and epoxyl resin constituents.

Objectives

The objectives of this research are to : (1) Use HPLC to develop characteristic profiles of aquatic fulvic acid and to identify similarities and differences between samples collected from different locations, (2) Compare the chromatographic characteristics of reference fulvic acid (purified water fulvic acid from Dr. Malcolm of the U.S. Geological Survey) with those studied in the first

objective, (3) Fractionate components of reference fulvic acid on a semipreparative scale using a suitable HPLC mode for further investigation by other spectroscopic methods.

For the first objective, aquatic fulvic acid extracted from water and sediment from several reservoirs and rivers in the southwest U.S. were used. For the second objective, standard and reference samples of purified fulvic acid were investigated by the same chromatographic modes used in the first objective. For the third objective, only Suwannee reference fulvic acid was used.

The approach used in this research is to optimize the fractionation of aquatic fulvic acid by altering the HPLC mode and the carrier solvent systems. Selection of the carrier solvent system is based on consideration of the presence of components ranging from non-polar to polar to ionic.

Further research on this topic includes the investigation of selected HPLC fractions by GC, GC-MS, Proton and Carbon-13 NMR and FAB Mass Spectrometry and other identification techniques. Furthermore, a limited study was conducted on the derivatization of fulvic acid in an effort to identify some of the structural details of these compounds.

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CHAPTER II

EXPERIMENTAL

Material and Methods

This research project involved the investigation of two sets of aquatic fulvic acid. One set of samples included fulvic acid extracted from water and sediment from different streams and lakes. The other set of samples included purified water fulvic acids that were provided by the U.S. Geological Survey. All samples were investigated by three different modes of HPLC namely, adsorption, weak anion exchange, and reversed-phase. Selected samples were investigated by Gas Chromatography/Flame Ionization Detector (GC/FID) after derivatization. Also, selected samples from semipreparative adsorption HPLC were investigated by Cross Polarization Magic Angle Spinning (CP-MAS) Carbon-13 NMR and Fast Atom Bombardment (FAB) Mass Spectroscopy. Fourteen organic model compounds were used as the retention references on the same GC or HPLC conditions. The selection of model compounds were made based on previous studies by Liao et al. (1), Saleh et al. (2), Schnitzer and Khan (3), Christman and Gjessing (4), and Reuter et al. (5).

Reagents

For Sample Extraction

HPLC grade water (CAS Reg. 7732-18-5), sodium hydroxide (CAS Reg. 1310-73-2) were obtained from Fisher Scientific Company. Nitric acid (CAS Reg. 7697-37-2) was purchased from MCB Chemical Company.

For HPLC

HPLC grade methanol (CAS Reg. 67-56-1), water (CAS Reg. 7732-18-5), acetonitrile (CAS Reg. 75-05-8), 1-butanol (CAS Reg. 71-36-3), as well as adipic acid (CAS Reg. 124-04-9), pyridine (CAS Reg. 110-86-1), o-cresol (CAS Reg. 95-48-7), ethylenediamine (CAS Reg. 107-15-3) and resorcinol (CAS Reg. 108-46-3) were obtained from Fisher Scientific Company. Tetrabutylammonium phosphate (CAS Reg. 42724-31-2) was obtained from Regis Chemical Company whereas sodium 1-dodecyl sulfate (CAS Reg. 151-21-3) was obtained from Alltech Associate Company. Acetic acid (CAS Reg. 64-19-7), sulfuric acid (CAS Reg. 7664-93-9) and nitric acid (CAS Reg. 7697-37-2) were purchased from MCB Chemical Company.

For GC

HPLC-GC/MS grade methylene chloride (CAS Reg.75-09-2) as well as N,N-dimethylformamide (CAS Reg. 6868-12-2), methyl iodide (CAS Reg. 74-88-4), adipic acid, pyridine, o-cresol, ethylenediamine and resorcinol were obtained from Fisher Scientific Company. HPLC grade hexane (CAS Reg.

110-54-3), ethyl ether (CAS Reg.60-29-7) were obtain from J.T. Baker Chemical Company. Methyl sulfoxide (CAS Reg. 67-68-5), octylamine (CAS Reg. 111-86-4), L-proline (CAS Reg. 147-85-3) and serine (CAS Reg. 56-45-1) were purchased from Eastman Kodak Company. Iodomethane-C-13 (CAS Reg. 74-88-4) was obtained from MSD Isotopes Company. Sodium hydride (CAS Reg. 7646-69-7), N-methyl-N-nitroso-p-toluenesulfonamide (CAS Reg. 80-11-5) and 2-(2-ethoxyethoxy) ethanol (CAS Reg. 37421-08-2) were obtained from Aldrich Chemical Company. DL-Aspartic acid (CAS Reg. 617-45-8), 2,4-dinitrophenol (CAS Reg. 51-28-5), 3,5-dinitrosalicylic acid (CAS Reg. 609-99-4), Salicylic acid (CAS Reg. 69-72-7) and L-alanine (CAS Reg. 56-41-7) were obtained from MCB Chemical Company. Furoic acid (CAS Reg. 88-14-2) was obtained from Matheson Coleman & Bell Chemical Company.

Sources of Sample

Surface water and sediment samples were collected from several reservoirs and rivers in the southwest of the U.S. The sampling sites were Lake Texoma, Oklahoma, Cross Lake, Louisiana, Lake Pat Mayse, Texas, and Red River, Texas. Surface waters were collected at less than one meter depth in 3.8-L brown glass bottles with Teflon-lined caps. Sediments were collected from the same water site using a Ponar grab sampler, depth of the sediment layer did not exceed 15 cm.

The purified Suwannee reference fulvic acid was provided by the U.S. Geological Survey. This purified fulvic acid was prepared by adsorption the water sample onto XAD-8 resin (6). This step can separate the humic substances from inorganic salts, free amino acids and free carbohydrates. Water-soluble organic acids, such as uronic acid and hydroxy acids, are also separated. After the precipitation of humic acid, the fulvic acid is freeze-dried.

Sample Preparation

Extraction of Fulvic Acid from Water

Water samples were filtered through a glass fiber filter (Schleicher & Schuell #30, 0.45 μm average pore size), purged with purified nitrogen for 30 minutes, and freeze-dried. The freeze-dried residue was solubilized in HNO_3 (pH 2) at a ratio of 10 mL/L of the original sample. The acidified residue was filtered and stored refrigerated. Table II shows the yield of the freeze-dried residues of each water sample.

Extraction of Fulvic Acid from Sediment

Dilute aqueous NaOH solution remains as one of the most commonly used reagent for effective extraction of humic substances from soils or sediments. There is some evidence that under alkaline conditions, autoxidation of humic constituents in contact with air may occur. Oxygen can be

TABLE II

THE YIELD OF RESIDUE CONTAINING WATER FULVIC ACID

Sample Site	Amount of Water	Residue Yield*	Residue Yield
	(L)	(g)	(g/L)
Lake Pat Mayse	5	0.4215	0.0843
Lake Texoma	3	2.7297	0.9099
Red River	3	0.3238	0.1079
Cross Lake	2	0.2130	0.1065

* Residue yield represent both the non-volatile organic and inorganic constituents in the water sample.

displaced from the soil-alkali system by bubbling an inert gas such as nitrogen into it, the container is filled with nitrogen and made air-tight (3).

Sediment fulvic acid was extracted by using the procedure of Stuermer and Payne (7), except for minor modifications (8). The sediment sample was sieved through 60 m particle size sieve. The % water composition was determined for each sediment sample. The mass of sediment used in the digestion was adjusted based on % water composition to yield a volume equivalent to 200 g dry weight of the sediment which was refluxed with 1000 mL 0.1 N NaOH under nitrogen atmosphere for 24 hours. The sample was acidified to pH 2 with 6 N HNO_3. The acidified residue was filtered through a glass fiber filter (SS #30). All the sediment digestion fulvic acids were stored refrigerated in a 3.8-L brown bottle.

Reference Fulvic Acid

All purified water fulvic acid samples were provided by Dr. Malcolm of the U.S. Geological Survey. These included fulvic acid from Suwannee River, Ogeechee River, Bear River, Ohio River, and Missouri River. The Ogeechee River samples included five separate samples collected at different dates.

Model Compounds

Fourteen organic model compounds were used as the retention references in order to correlate their behavior with those fulvic acid components. Table III shows the model compounds and their structure. All the model compounds were prepared at the concentration of 1 mg/5 mL HPLC water for HPLC and GC studies. The model compounds were also prepared in methylene chloride for use in a GC capillary column.

Sample Preparation for HPLC

All water fulvic acid samples were prepared from the freeze-dried residue of each water sample at the concentration of 1 mg/5 mL HPLC water. The sediment fulvic acids were prepared from the sediment digestion. The reference fulvic acids and model compounds were prepared at the concentration of 1 mg/5 mL HPLC water.

A 70 mg Suwannee River reference fulvic acid was used for fractionation by semipreparative RP-HPLC. Batches of 10 mg of the fulvic acid were dissolved in 1 mL HPLC water. The fulvic acid was fractionated into two fractions using stepwise gradient conditions with the first solvent A1 (1% MeOH and 99% water) and the second solvent B1 (85% MeOH and 15% water). Each fraction was then freeze-dried, desiccated and weighed to a constant weight. Each fraction was reinjected on the RP-HPLC column to check on the efficiency of fractionation.

TABLE III

MODEL COMPOUNDS AND THEIR STRUCTURES

	Compound	Structure
1	Resorcinol	$C_6H_4(OH)_2$ (1,3-dihydroxybenzene ring: OH, OH)
2	Vanillic acid	HOOC–ring–OH, OCH_3
3	Adipic acid	$HOOC(CH_2)_4COOH$
4	Alanine	$CH_3CH(NH_2)COOH$
5	2,4-Dinitrophenol	ring: OH, NO_2, NO_2
6	Proline	$H_2C(CH_2NH)(CH_2CHCOOH)$ ring
7	Aspartic acid	$HOOC\,CH_2\,CH(NH_2)COOH$
8	Salicylic acid	ring: OH, COOH
9	Furoic acid	furan ring (O)–COOH
10	3,5-Dinitrosalicylic acid	ring: COOH, OH, O_2N, NO_2
11	Serine	$HOCH_2\,CH(NH_2)\,COOH$
12	Pyridine	ring with N
13	O-Cresol	ring: CH_3, OH
14	Ethylenediamine	$H_2N\,CH_2\,CH_2\,NH_2$

Sample Preparation for GC

Direct injection. The water and sediment fulvic acids were prepared as for HPLC. The reference fulvic acids were prepared at the concentration of 1 mg/ 5 mL. All the model compounds were prepared at the concentration of 1 mg/ 5 mL in HPLC water and 1 mg/ 1 mL in methylene chloride.

Extraction after derivatization. Fulvic acid was methylated by using the procedures similar to Wershaw and Pinckney (9) and Wershaw et al. (10). The methylation was divided into two steps. In the first step, carboxylic acid groups were methylated with diazomethane in N,N-dimethylformamide (DMF); in the second step, hydroxyl groups were methylated with methyl iodide and NaH in DMF.

The fulvic acids were methylated with diazomethane respectively. The apparatus for this methylation step is shown in Figure 4. A 20 mg sample of Suwannee River and Ogeechee river reference fulvic acids was dissolved in 1 mL DMF. Five mL of ethyl ether was added to the first tube of the apparatus to saturate the nitrogen carrier gas with ether. Then 0.7 mL of ether, 0.7 mL of carbitol, 2-(2-ethoxyethoxy) ethanol, 1 mL of 37% aqueous KOH, and 0.1-0.2 g of N-methyl-N-nitroso-p-toluenesulfonamide ("Diazald", Aldrich Chemical Co.) were added to the second tube. The base immediately began to release diazomethane from the

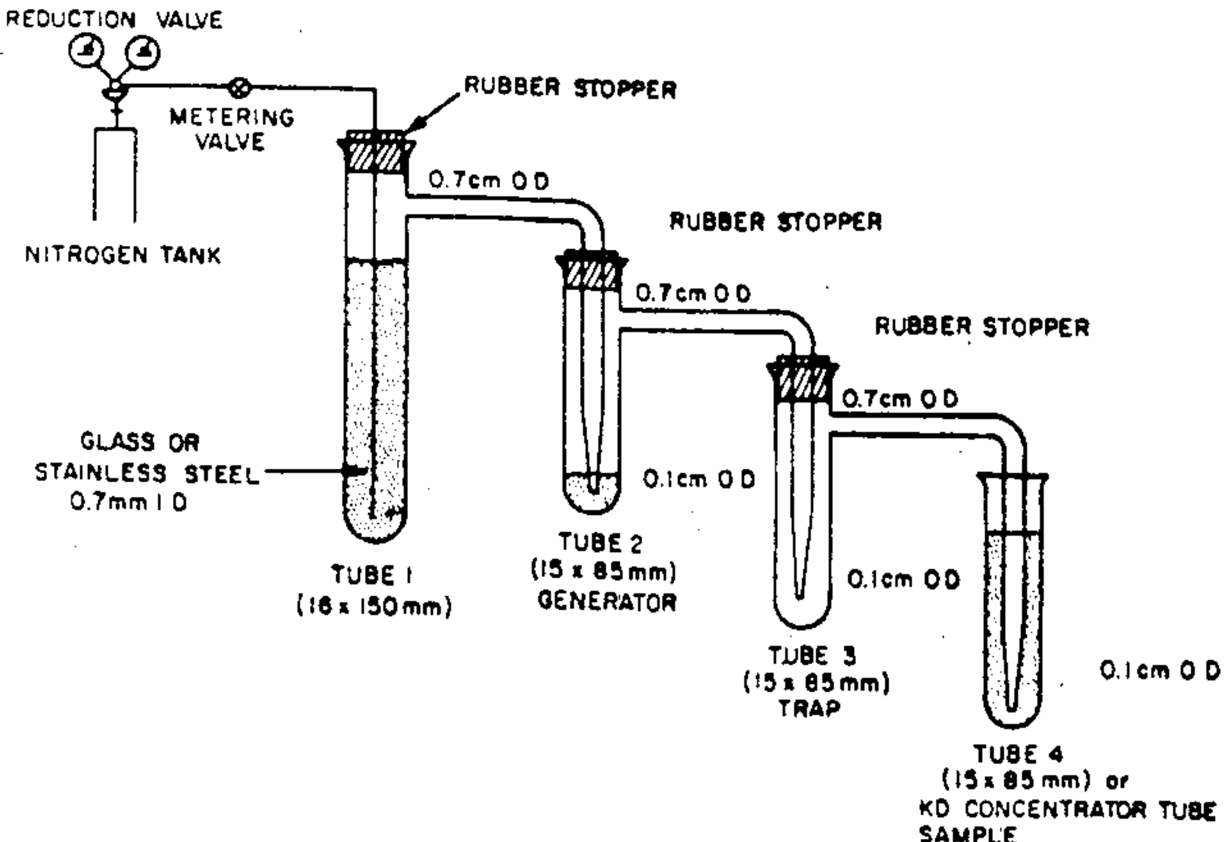

Figure 4--Apparatus for diazomethane methylation.

sulfonamide. Immediately position the second test tube and adjust the nitrogen flow to about 10 mL per minute. Then positioned the third tube (a safety trap) and the sample tube to bubble the nitrogen and diazomethane gas mixture through the sample. The reaction was continued about 30 minutes until the slight yellow color of diazomethane persisted in the sample solution. The products from this reaction were recovered by freeze-drying and redissolved in DMF.

The next methylation step was to add a 10-fold excess of sodium hydride (NaH) and methyl iodide (CH_3I) to this solution. The apparatus for this step is shown in Figure 5. About 200 mg of NaH powder and 10 mL hexane was added into the flask, stirred and purged with nitrogen gas for 10 minutes. Then remove hexane with a long needle syringe, then add 1 mL of methyl iodide. Sample fulvic acid was added into the flask drop by drop. At the end of the reaction period, the solution was poured into 15 mL of water, acidified with 30% hydrochloric acid to pH 2. The solution was extracted with 5 mL methylene chloride four times and the water layer was discard. The methylene chloride solution was washed 5-6 times with water to remove the DMF and evaporated to dryness on a hot water bath. The derivatized fulvic acids were prepared in methanol, ethylene chloride, hexane and water for the further studies.

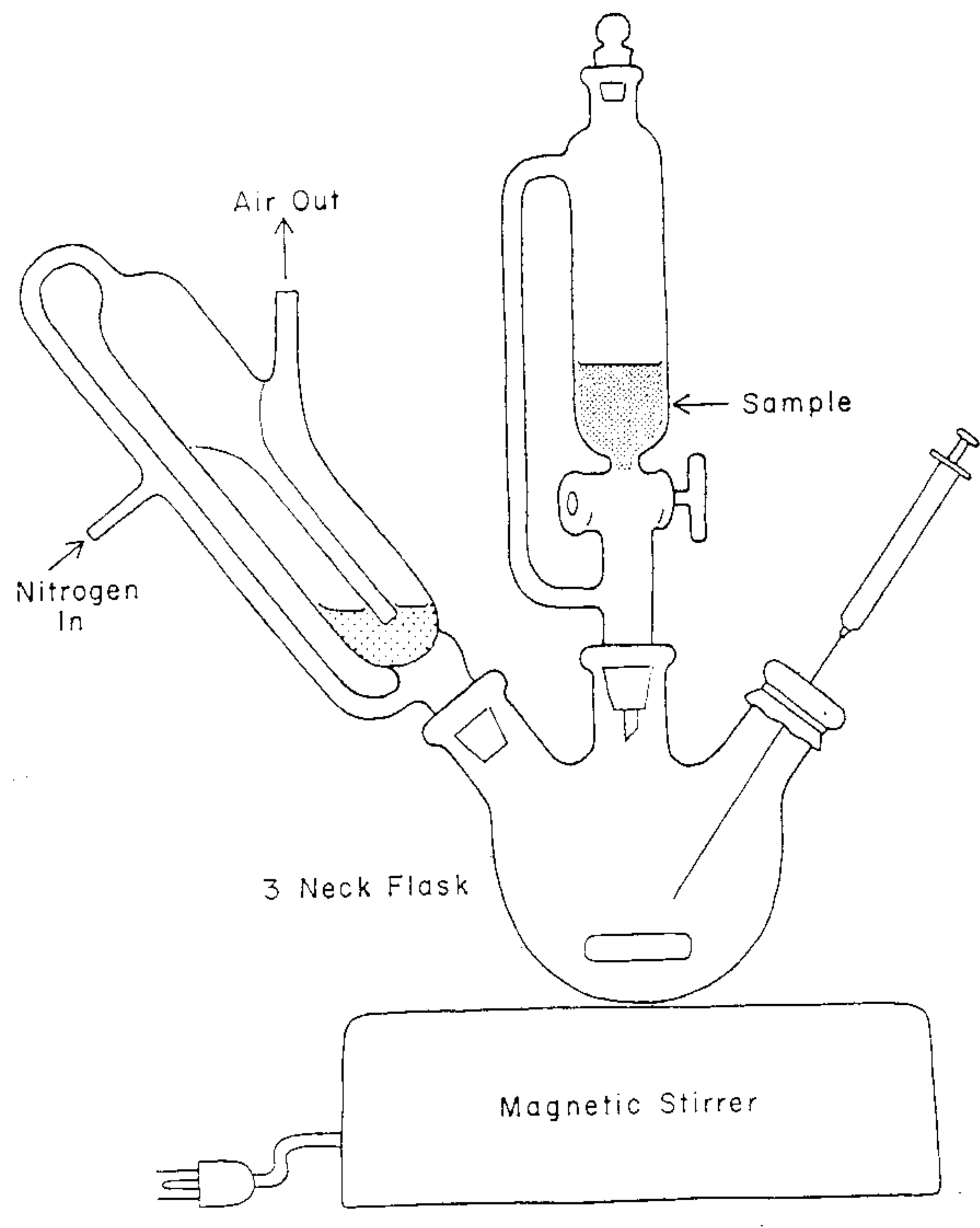

Figure 5--Apparatus for methylation with sodium hydride and methyl iodide.

Instrumentation

High Performance Liquid Chromatography System

The HPLC instrument was Waters ALC-201 with a Model 6000 pump and a 6-port standard Valco sampling valve equipped with a 25-μL, 100-μL as well as 1-mL loops. Both UV (Model ISCO Au-5) or (Beckman Model 160) and fluorescence detectors (Schoeffel Model 970) were simultaneously used to monitor the HPLC fractionation.

Three modes of HPLC were used in this study. One is adsorption, the second is anion exchange and the third is reversed-phase HPLC.

Adsorption HPLC. The column used in this HPLC mode was semipreparative stainless steel column (50 cm length x 8 mm i.d.) packed with methyl methacrylate resin (Johns Manville, Chromosorb 108 of 110-120 mesh). A 200-mL sample of Cross Lake sediment FA (corresponding to 20 g dry weight of the sediment) was fractionated into two fractions, using acetic acid at pH 3.1 and ammonium hydroxide at pH 11.7 under stepwise gradient conditions. The eluant was simultaneously monitored by the UV detector set at 254 nm and the fluorescence detector was set at λex 273 nm and λem 387 nm. The solvents were removed by freeze-drying. Acidic solutions (HNO_3, pH 2.2) of the fractions were reinjected on the column to check the separation efficiency. The freeze-dried residues of these two HPLC separated fractions

and the unfractionated residue were subjected to CP-MAS Carbon-13 NMR and FAB mass spectroscopy.

Anion exchange HPLC. The column used was a stainless steel column (100 cm length x 4 mm i.d.) packed with WAX ID. The Cross Lake sediment and reference fulvic acids were fractionated into three fractions by using acetic acid at pH 3.1 and acetic acid with triethylamine at pH 6.9 under stepwise gradient conditions.

Reversed-phase HPLC. Four reversed-phase analytical columns were used (1) 10 μm Partisil-10 ODS (C_{18}, Whatman), (2) 10 μm Rsil Phenyl (C_8, Alltech), (3) 5 μm Nova Pak (C_{18}, Waters), (4) 25 μm ST/C_{18} (C_{18}, Separations Technology). All columns except the Nova Pak column were packed in-house. Three analytical stainless steel columns (25 cm length x 4 mm i.d.) were packed with Partisil-10 ODS, ST/C_{18} and Rsil Phenyl. Two preparative stainless steel columns (60 cm length x 8 mm i.d. and 50 cm length x 22.5 mm i.d.) were packed with 25 g and 98 g of ST/C_{18}, respectively.

Two pairs of carrier solvent were used in an isocratic condition and five pairs of solvent were used in a stepwise gradient condition. Table IV shows the composition of these solvent systems. These solvent systems included polar and less-polar solvents in the presence or absence of acetic acid and ion pairing or organic modifiers. The different

TABLE IV

CARRIER SOLVENT COMPOSITION

Solvent System	Composition				
	Water	:	Acetic Acid	:	1-Butanol
Solv. II	347	:	1	:	11
	Water	:	Acetonitrile		
Octylamine 0.01M	85%	:	15%		
	Water	:	Acetic Acid	:	Methanol
Solv. A	98.9%	:	0.1%	:	1%
Solv. B	14.9%	:	0.1%	:	85%
	Water	:	Acetic Acid	:	Acetonitrile
Solv. C	98.9%	:	0.1%	:	1%
Solv. D	14.9%	:	0.1%	:	85%
	Water	:	Methanol		
Solv. A1	99%	:	1%		
Solv. B1	15%	:	85%		
	SDS*	:	Sulfuric Acid	:	Methanol
SDS-A	99.99%	:	0.01%	:	0%
SDS-B	52.49%	:	0.01%	:	47.5%
	PICA**	:	Methanol		
PICA	100%	:	0%		
PICA-M	75%	:	25%		

The first two carrier solvent systems were used under the isocratic condition, the other solvent systems

TABLE IV--Continued

were under stepwise gradient condition.

* SDS : Sodium 1-Dodecyl Sulfate 0.005 M Aqueous

** PICA : Tetrabutylammonium Phosphate 0.005 M Aqueous

solvents were used to develop characteristic fingerprints for the samples. A selected carrier solvent was then used for the semipreparative separation. To optimize the utilization of UV and fluorescence detectors several UV absorption and fluorescence excitation and emission wavelengths were used for on set of samples. Five combinations of different fluorescence excitation and emission wavelengths were compared using Solv. II system under an isocratic condition. Also five combinations of fluorescence excitation and emission wavelengths and three combinations of UV wavelengths were compared by using SDS-A and SDS-B solvent system under stepwise gradient condition.

Gas Chromatography System

Two gas chromatographs were used in this study. A Hewlett Packard 5710A gas chromatograph employing a flame ionization detector was used. The columns used were 6 ft x 7/32 in. o.d. (1.60 mm i.d.) glass tubes packed with (a)10% SP-2100 and (b)1% SP-1240 DA on 100-120 mesh Supelcoport (Supelco, Inc.). The injector and detector were maintained at temperatures of 250 and 300 °C, respectively. The temperatures of columns were programmed at 4 °C/min from an initial temperature set at 70 °C to a final temperature of 200 °C for 4-min delay for SP-1240 DA column. The temperatures of SP-2100 were programmed at (a)4 °C/min and (b)2 °C/min after a 4-min delay from an initial temperature

set at 80 °C to a final temperature of 240 °C for 16-min stay. The flow of helium carrier gas was 30 mL/min. All the sediment, water, reference fulvic acid and model compounds were injected and analyzed by the SP-1240 DA column. For the SP-2100 column, the temperature of the column was programmed at 4 °C/min from an initial temperature set at 80 °C to a final temperature of 240 °C. The samples injected at this condition were the derivatized fulvic acid dissolved in methanol, water and DMF. The sediment and reference fulvic acids were injected under the different conditions. The column was programmed at 2 °C/min from an initial temperature set at 80 °C to a final temperature of 240 °C and kept at 240 °C for 16 minutes.

The second GC used was a Varian Vista 6000 gas chromatograph equipped with flame ionization detector. A SE-30 capillary column was used. The injector and detector were maintained at temperatures of 250 and 300 °C, respectively. The temperature of column was programmed at 5 °C/min from an initial temperature set at 50 °C to a final temperature of 200 °C for 10-min stay. The flow of helium carrier gas was 30 mL/min. All the sediment and water fulvic acids were injected. The reference fulvic acid was dissolved into methanol, methylene chloride and hxane so that resolution could be compared.

CP-MAS Carbon-13 NMR System

The Carbon-13 NMR work was done by the scientists at the Colorado State University Regional NMR center. The description given for this instrument was approved by the center.

Carbon-13 NMR spectra were obtained with a Nicolet NT-150 spectrometer at 37.7 MHz using a home built CP-MAS modification including the probe. The cross polarization of contact time was 1 ms and the pulse repetition time was 1 s. The ^{1}H irradiation field was 11G and 1K data points were zero filled to 2K points in the spectra. Chemical shifts were measured with respect to tetramethylsilane via hexamethylbenzene as a secondary substitution reference (aromatic peak at 132.3 ppm). Usually 15000-50000 scans were accumulated. Bullet-shaped spinners (11) were used with sample volume of 0.4 cm and were spun at about 3.8 kHz.

FAB Mass Spectroscopy System

The FAB-MS work was done by scientists at the Johns Hopkins Regional Instrumentation Facility. The description given for this instrument was approved by the center.

FAB mass spectra were obtained on a Kratos MS-50 instrument equipped with 23 kG magnet which extends the mass range to 3000 amu at the full accelerating voltage of 8 kV. Spectra were recorded oscillographically at low resolution

with an accelerating potential of 4 kV and scan rate of 100 s/decade. The pressure in the ion source housing was maintained within the 10^{-6} torr region. An argon beam of 4-6 keV produced from an FAB source was impacted into the sample dissolved in glycerol. FAB spectra were obtained in the positive ion mode. The method of sample introduction was similar to that described by Grigsby et al. (12). A copper target on the end of a direct-introduction probe was cleaned with nitric acid, rinsed with distilled water, and dried with a paper towel. One drop of glycerol was placed on the target and ~1 mg of sample was added from the end of a 1 mm o.d. capillary tube. The mixture was then stirred with the tube to form a film of solution over the face of the target. After the sample was degassed for ~ 1 min in the vacuum lock of the mass spectrometer, the probe was inserted into the ion source, the high voltage was turned on, and the spectral recording was started.

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CHAPTER III

RESULTS AND DISCUSSION

The results and discussion are presented in four sections. Essentially three modes of HPLC were applied to aquatic fulvic acids from different sources and locations. First, the results of adsorption HPLC on Cross Lake sediment fulvic acid are presented. This section also includes the semipreparative fractionations of Cross Lake fulvic acid followed by Carbon-13 NMR and FAB Mass Spectroscopy. Second, the results of anion exchange HPLC on Cross Lake sediment fulvic acid are presented. This section includes the anion exchange HPLC fractionation followed by reversed-phase HPLC. Third, the results of reversed-phase HPLC are presented. These include that RP-HPLC of fulvic acids from different locations as well as reference fulvic acids are compared. Several types of carrier solvent were used to evaluate the fractionation. One set of carrier solvent was selected to fractionate reference fulvic acid in a semipreparative scale. Finally, the results of application of gas chromatography are presented. These include a two-step methylation procedure for fulvic acid.

Adsorption HPLC

Adsorption (liquid-solid) chromatography involves a liquid mobile phase plus a finely divided, porous solid as a stationary phase (adsorbent). The adsorbent should have a relatively large specific surface area. The equilibrium that governs separation is based on the distribution of sample molecules between the bulk liquid phase and the surface of the adsorbent. The effect of the adsorbent on separations by adsorption chromatography is determined by several adsorbent properties, primary chemical type, surface area, and water content. The polar adsorbents interact with adsorbed molecules via specific forces such electrostatic attraction and hydrogen bonding. Dispersion interactions tend to cancel and hence are less important in determining relative adsorption (1). Adsorption chromatography is generally considered to be suitable for the separation of non-ionic molecules which are soluble in organic solvents and moderately polar compounds (2).

The mechanism by which retention occurs in adsorption chromatography is not understood with absolute certainty. Two models of the adsorption process have been proposed: (a) the competition model developed by Snyder and Soczewinski (3, 4) and (b) the solvent interaction model proposed by Scott and Kucera (5). For nonpolar and moderately polar mobile phases which interact with the adsorbent surface largely by dispersive and weak dipole

interactions, the competition model assumes that the entire adsorbent surface is covered by a monolayer of solute and mobile phase molecules. Under normal chromatographic conditions, the concentration of sample molecules are small and the adsorbed monolayer will consist mainly of mobile phase molecules. Retention of a solute molecule then occurs by displacing a roughly equivalent volume of mobile phase molecules from the monolayer to make the surface accessible to the adsorbed solute molecule.

The solvent interaction model differs from the competition model by proposing the formation of solvent bilayers adsorbed onto the adsorbent surface. The composition and extent of bilayer formation depends on the concentration of polar solvent in the mobile phase. Solute retention occurs by interaction (displacement or association) of the solute with the second layer of adsorbed mobile phase molecules (2).

Retention and selectivity in adsorption chromatography are dramatically influenced by the presence of even low concentrations of polar additives in the mobile phase. These additives, known as moderators or modulators, exert an overriding influence on the separation properties of the chromatographic system. The most ubiquitous example is water. The influence of this moderator is most pronounced when the mobile phase is nonpolar. In addition to water, virtually any organic polar modifier may be used to control solute retention in adsorption chromatography.

Fractionation of Fulvic Acid

Adsorption chromatography using Chromosorb-108 column was used in earlier phase of this study which was a continuation of earlier work by Saleh and Mokti (6). Three solvent pairs were used in a stepwise gradient mode in that study. The first solvent pair was H_3BO_3 at pH 3.75 and NaOH at pH 11.70. The second solvent pair was CH_3COOH at pH 3.02 and NH_4OH at pH 11.70. The third solvent pair was HNO_3 at pH 2.0 and NaOH at pH 11.70. Results indicated that pH profile of the carrier solvent system and the extent of protonation of the fulvic acid macromolecule control the fractionation on Chromosorb-108 resin. Figure 6 shows a typical adsorption HPLC chromatogram of fulvic acid extracted from Cross Lake sediment. Peaks I and II were collected on a semipreparative scale by using Chromosorb-108 column with acetic acid (pH 3.1) and ammonium hydroxide (pH 11.7) as the carrier solvents. It is noted that the area under peak II represents $\simeq$ 80% of the u.v. absorption signals and $\simeq$90% of the fluorescence signals. Table V shows molecular size distribution data of the total sample and fractions.

CP-MAS Carbon-13 NMR

Residues collected from peak I and II were subjected to CP-MAS Carbon-13 NMR and FAB Mass Spectroscopy. Results are reported in a publication by Saleh et al. (7). Figure 7 shows the CP-MAS Carbon-13 NMR spectrum of the freeze-dried

Fluorescence Response λ_{ex} 273nm λ_{em} 387nm

UV Response λ 254nm

Figure 6--C-108 chromatogram of fulvic acid extracted from Cross Lake sediment (solid lines) and procedure blank (dashed lines); solvent (1) acetic acid at pH 3.1; solvent (2) NH_4OH at pH 11.7 (from ref. 7).

TABLE V

APPARENT MOLECULAR WEIGHT DATA* OF TOTAL AND FRACTIONATED AQUATIC FULVIC ACID (FROM REFERENCE 7)

	Total Sample	Fraction I	Fraction II
wt av mol wt "Mw"	3.93×10^3	1.8×10^3	6.05×10^3
no. av mol wt "Mn"	1.93×10^3	0.8×10^3	2.36×10^3
range of mol wt	31.62×10^3	7.08×10^3	28.18×10^3
	to	to	to
	0.18×10^3	0.09×10^3	0.14×10^3

* Determined by Size exclusion HPLC

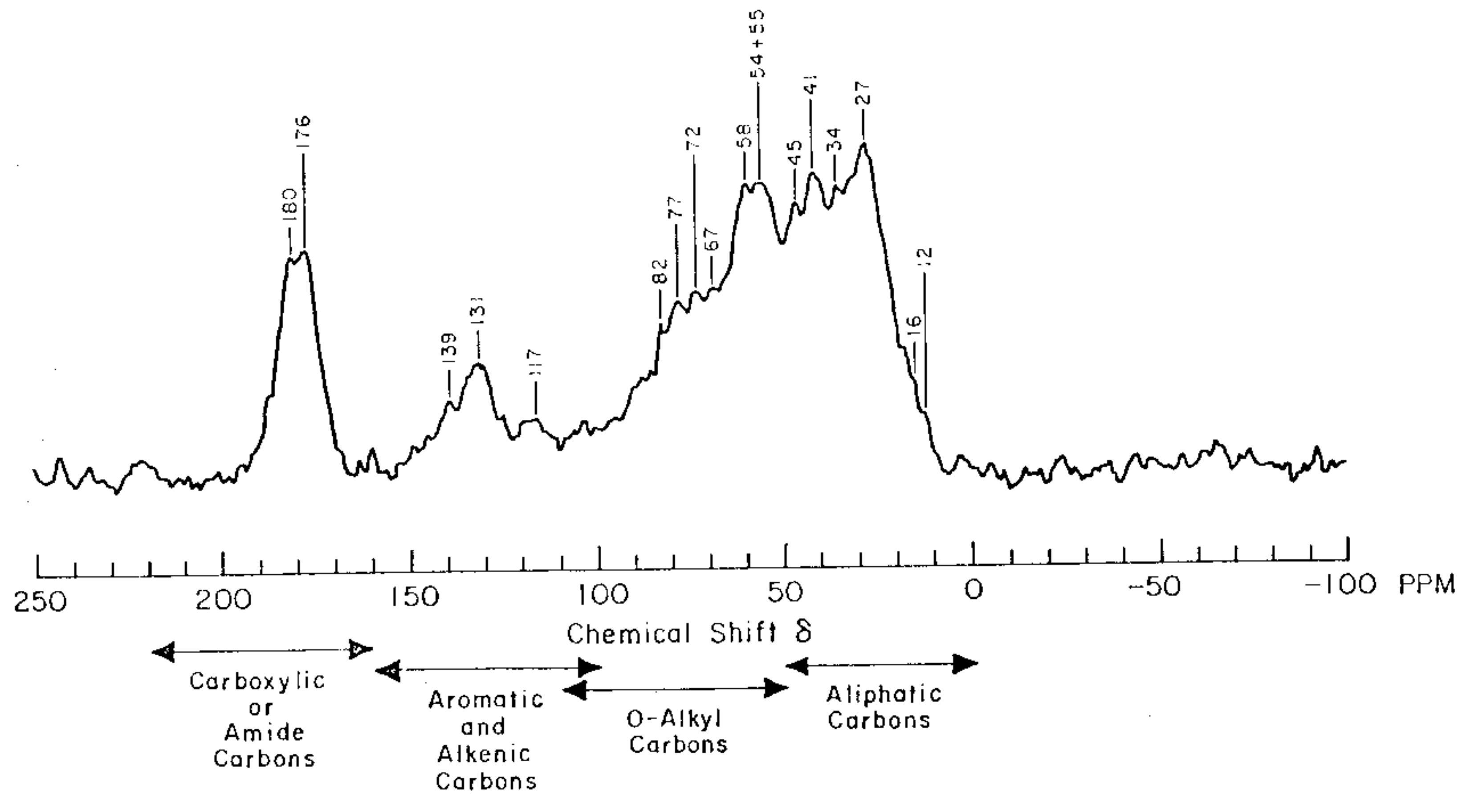

Figure 7--CP-MAS Carbon-13 NMR spectrum of fractionated fulvic acid, 25000 scans (from ref. 7).

residue (0.220 g) of peak II in Figure 6. Three resonance envelops are characteristic of the spectrum. The chemical shift in the region between δ 0 and 70 ppm, corresponds to aliphatic carbons. The chemical shift in the region between δ 100 and 160 ppm corresponds to aromatic, heteroaromatic, and olefinic carbons. The region between 170 and 190 ppm corresponds to carbons in the carboxyl or amide groups. To interpret the CP-MAS Carbon-13 NMR spectra, two questions must be addressed. First, are all the carbons in the sample cross polarized equally so that all the carbon types contribute proportionately to the integrated intensities ? Second, what types of interferences, if any, may arise from other components of the sample ? The answer to these questions is partly dependent on factors related to the structure of the organic material (e.g., aromaticity, ring size, tertiary and quaternary carbons) and experimental factors (contact time and pulse repetition rates). Several workers (8, 9, 10, 11, 12) have discussed provisions in interpretation of CP-MAS Carbon-13 NMR spectra of complex organic materials. In spectra of such material, there is a considerable overlap between signals from different types of carbons. Also, uncertainty may result from effects of paramagnetic material of signal intensities and line widths. Nevertheless, qualitative information on the different types of carbon can be derived from the spectra. With

semiquantitative estimates, it may be possible to draw conclusions regarding the relative abundance of the various carbon types.

Table VI summarizes the chemical shifts and structural assignments. The spectrum reveals prominent signals in the alkyl region extending from δ 10 to 50 ppm. Intensities are defined by peaks at δ 27, 34, and 45 ppm. These signals typify aliphatic side chain carbons and carbons in α, β, or γ position from terminal methyl group or an aromatic ring. The shoulders at δ 10-16 ppm arise from terminal methyl groups. The o-alkyl region extends from δ 50 to 100 ppm. The rather strong signals at 54 and 55 ppm can be assigned to methoxy carbon. Ether and carbohydrate carbons resonate at δ 60-72 ppm and at 101 ppm and small amounts of these materials may be present. The aromatic carbons extend over the region from 101 to 160 ppm. The three peaks at δ 117, 131 and 139 in this region suggest the presence of different types of substituted aromatic structures.

The general configuration of the Carbon-13 NMR spectrum is similar to the one reported by Hatcher et al. (13) for soil fulvic acid, except in the resonance region between δ 60 and 100 ppm which indicates higher alcoholic and carbohydrate carbons in soil fulvic acid. Apparently, aquatic fulvic acid contains relatively few of these carbons. It is also possible that these components did not

TABLE VI

CHEMICAL SHIFTS AND STRUCTURAL ASSIGNMENTS[a] IN THE CP-MAS CARBON-13 NMR SPECTRUM OF FULVIC ACID (FIGURE 7) (FROM REFERENCE 7)

chemical shift region	obsd resonance peaks, ppm	range of chemical shift, ppm	assignment
aliphatic carbons (0-50 ppm)	12-16	13-17	terminal methyl carbon or methyl carbon γ or further aromatic ring
	27-34	19-35	methyl carbon α to aromatic ring or methylene carbon β, γ, or δ from terminal methyl group
	11-45	37-53	methylene carbons of branched alkyls
oxygen alkyl carbons (50-110 ppm)	54, 55	54-56	methoxy carbons
	58, 67, 72, 77, 82	60-101	carbon atoms in polyhydroxy compounds and certain amino acids
aromatic and alkenic carbons (100-160 ppm)	117	108-118	aromatic carbon ortho to ether oxygen or hydroxy group
	131, 139	118-145	aromatic and substituted aromatic carbons
carboxylic, carbonyl, or amide carbons (160-220 ppm)	176, 180	150-196	carbon in carboxylic acids, esters, or amides

[a] Some overlap of shift ranges is likely.

elute with fraction of fulvic acid. Peaks corresponding to methoxy carbons are more pronounced in the fractionated aquatic fulvic acid than in soil fulvic acid. Similarity between the Hatcher's fulvic acid spectrum and the current spectrum lies essentially in the predominance of aliphatic moieties over the aromatic ones. Also, as noted by Hatcher et al. 1981, phenolic carbons which resonate between δ 148 and 153 ppm are not detected in the Carbon-13 NMR spectra. Hatcher et al. offered two possible explanations to this observation. The first is the possibility of overestimation of phenolic compounds in fulvic acid, using chemical methods. The second is the possible masking of the phenolic carbons due to the stable free radicals in the sample. We believe that the second explanation is more likely. Presence of stable free radicals in fulvic acid and humic material have been reported by several workers using electron spin resonance (ESR) spectrometry (14, 15, 16, 17, 18, 19). Later ESR work at NTSU, on the fractionated fulvic acid sample confirmed the presence of free radicals (20).

The CP-MAS Carbon-13 NMR spectra on the freeze-dried residue of the total sample and the fraction corresponding to peak I in Figure 6 did not reveal measurable signals above the noise level possibly because of the relatively low carbon content of the sample and the presence of additional paramagnetic ions.

Fast Atom Bombardment Spectra

The same sample subjected to CP-MAS Carbon-13 NMR was subjected to FAB mass spectrometry. Figures 8 and 9 show the FAB spectra of the sample dissolved in glycerol and in glycerol plus sodium chloride. Addition of Na^+ is known (21) to enhance the production of cationized species $(M + Na)^+$ and reduce, to a large extent, the production of fragment ions.

Unexpectedly, no high molecular weight ions (above m/z 300) were detected in either FAB mass spectra. Several of the small molecular weight ion were tentatively identified. Figures 10-12 show the major molecular ions and the possible molecular formulas and structures. Of special interest is the shift of peaks at m/z 125, 127, 154, and 183 in Figure 8 to m/z 147, 149,176, and 205 in Figure 9 which correspond to the $(M + Na)^+$ species. Easily cationized species are expected to be more prominent in the FAB spectra than those of nonpolar molecules such as aliphatic hydrocarbons. However, Barber et al. (22), reported FAB of mixture aliphatic hydrocarbons consisting of n-$C_{22}H_{46}$, n-$C_{20}H_{42}$, n-$C_{18}H_{38}$, and n-$C_{16}H_{34}$. There are some indications for the presence of aliphatic structures such as those aliphatic hydrocarbons in the FAB mass spectra of fractionated fulvic acid as detected in Figure 8 by signals at m/z 115, 129, 143, 157, 227, and 255 which correspond to structures X.

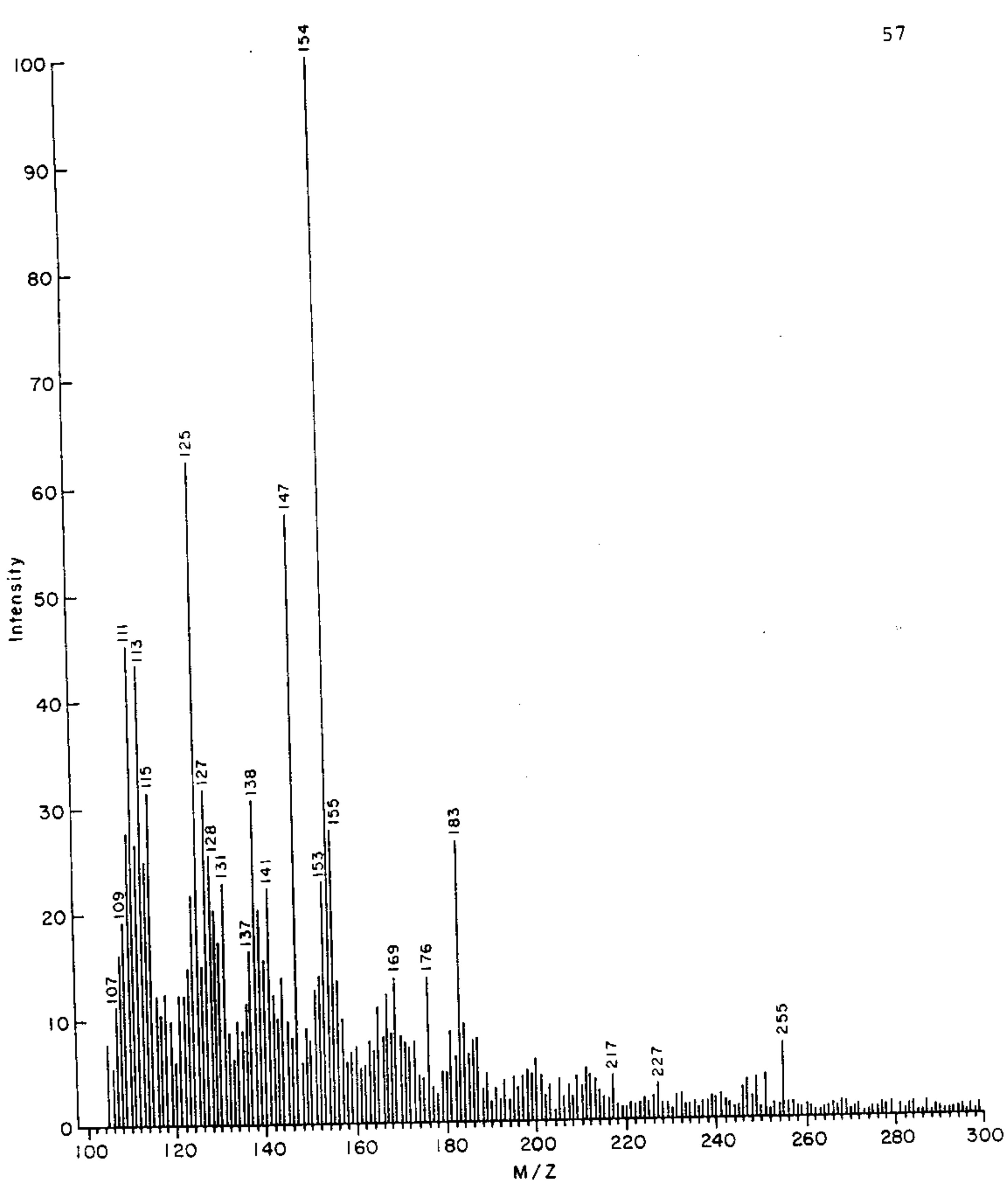

Figure 8--FAB mass spectrum of fractionated fulvic acid dissolved in glycerol (from ref. 7).

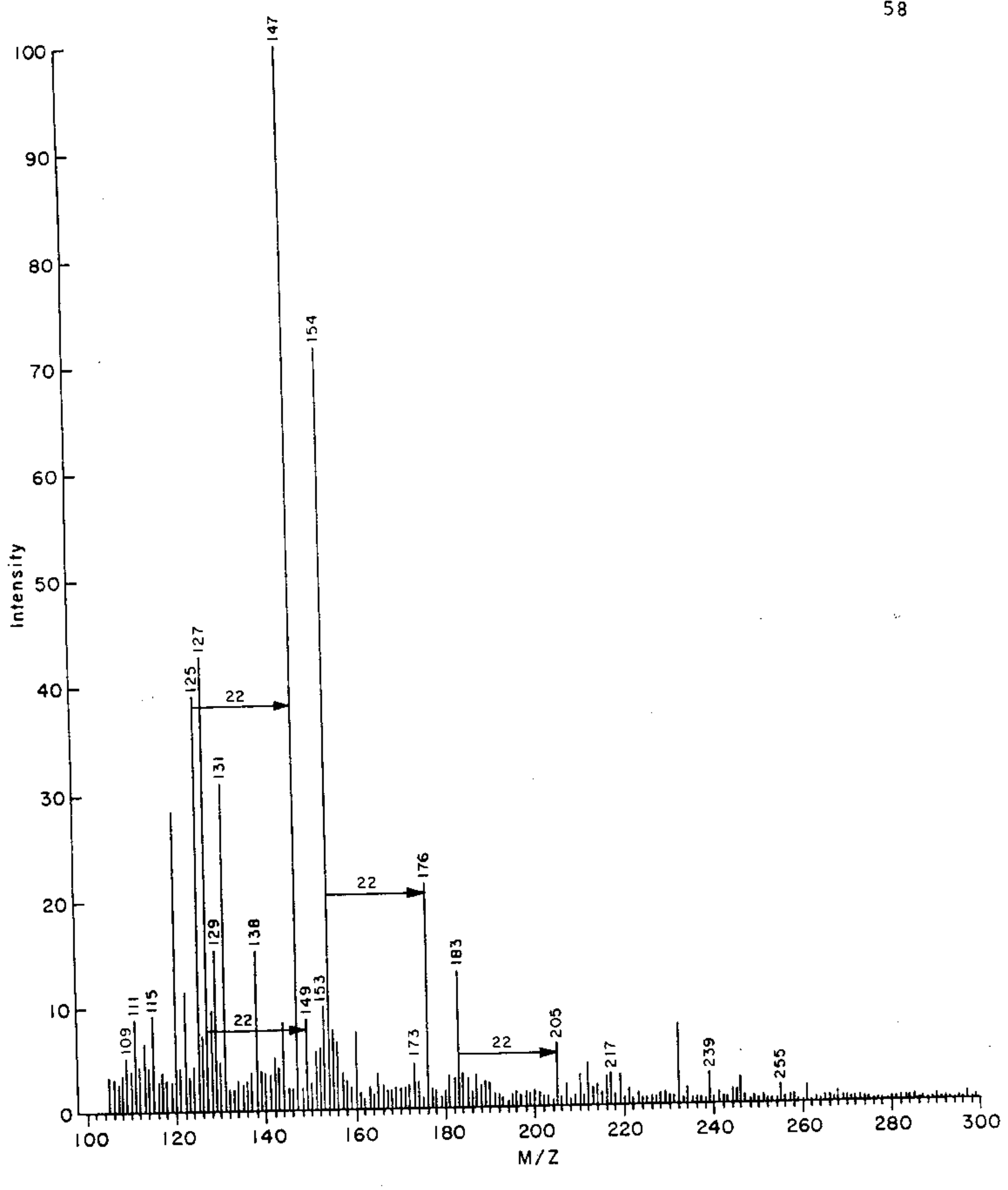

Figure 9--FAB mass spectrum of fractionated fulvic acid dissolved in glycerol, NaCl added (from ref. 7).

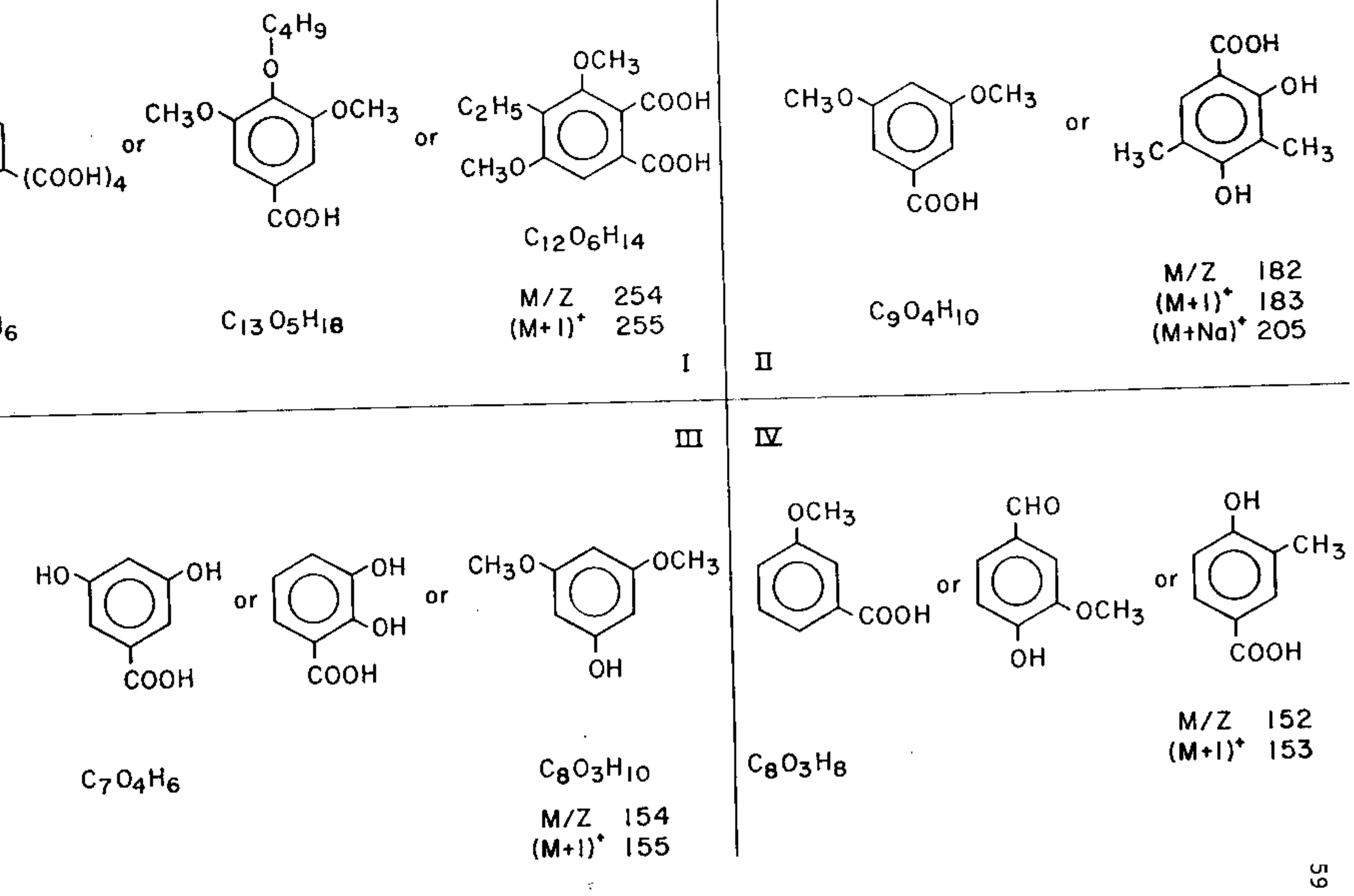

Figure 10--Possible molecular ions in FAB mass spectra of fractionated fulvic acid (from ref. 7).

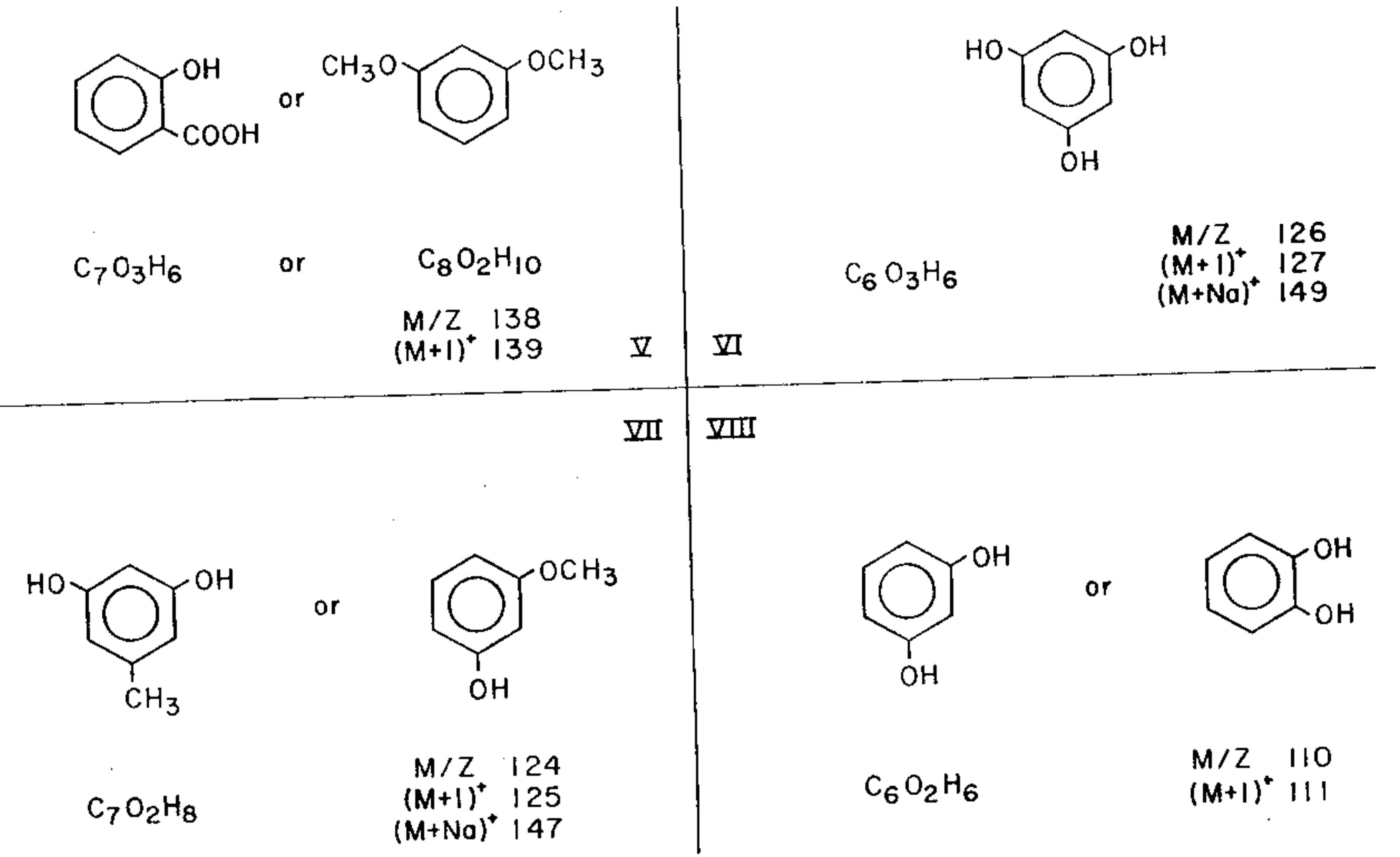

Figure 11--Possible molecular ions in FAB mass spectra of fractionate fulvic acid (from ref. 7).

IX

$C_6H_5OCH_3$ or $C_6H_4(OH)(CH_3)$

C_7OH_8

M/Z 108
$(M+1)^+$ 109
$(M+Na)^+$ 131

X

C_nH_{2n+2}

n	M/Z	$(M+1)^+$
8	114	115
9	128	129
10	142	143
11	156	157
16	226	227
18	254	255

XI

H–C–(CH_2) COOH ‖ H–C–COOH and H–C–$(CH_2)_2COOH$ ‖ H–C–$(CH_2)_2COOH$

$C_5O_4H_6$

M/Z 130
(M+1) 131
(M+Na) 153

$C_8O_4H_{12}$

M/Z 172
(M+1) 173

XII

$(CH_2)_4(COOH)_2$ and $(CH_2)_9(COOH)_2$

$C_6O_4H_{10}$

M/Z 146
$(M+1)^+$ 147
$(M+Na)^+$ 169

$C_{11}O_4H_{20}$

M/Z 216
$(M+1)^+$ 217
$(M+Na)^+$ 239

Figure 12--Possible molecular ions in FAB mass spectra of fractionated fulvic acid (from ref. 7).

Also, signals of m/z 131, 147, 173, and 217 which are present in both spectra may be identified as aliphatic dicarboxylic acids (structures XI and XII), in Figure 12. Polymaleic acid has been suggested (23, 24) as one of the building blocks of humic material. It should be emphasized that structures presented in Figures 10-12 are only speculative. Background information from the CP-MAS Carbon-13 NMR spectrum and from published data (25, 26) on chemical degradation studies of fulvic acid augmented the assignment of these structures. The identified structures are potential precursors to THMs upon chlorination (27) and contain complexing sites for metals (28).

Theoretically, FAB mass spectrometry offers the ideal technique for studying polar high molecular weight compounds such as fulvic acid. The reason why no high molecular weight ion were detected in the fractionated fulvic acid was unknown at that time and only speculations could be made. The mechanisms of molecular and fragment ion production were not established in detail. One possible explanation for the lack of detection of high molecular weight ions is that the fulvic acid macromolecule is formed of aggregates of small molecules that are held by different types of bonding. The generation of a hot spot is a central feature of FAB-MS and energy is dissipated in the sample substrate by generation of a collisional cascade which results in a thermally

activated region with a core containing temperatures near 10^4 K (29). Under such conditions the fracture of the several types of bonding is quite possible. The forementioned explanation supports the aggregates model of fulvic and humic acids, reported by some investigators (30, 31).

Barber et al. reported that the fragment ions present at reasonable abundance in most FAB mass spectra have been shown to arise, in part, by gas-phase unimolecular decomposition reaction steps originating with molecular ionic species. Considering this explanation, one might select certain structures in Figures 10-12 that indicate unimolecular decomposition reactions. This was one of the first reporting of FAB mass spectra of fulvic acid and further investigation of this type of compound by FAB-MS and modification of the instrumental conditions may reveal more details of the mechanisms pertinent to the interpretation of FAB mass spectra of humic material.

The FAB-MS were run on the freeze-dried residue of the total and fraction corresponding to peak I in Figure 6. The highest molecular ion occurred at m/z 391. The spectra were uninterpretable due to the very low intensity of the characteristic molecular ions and fragments and the predominance of masses $(92n + 1)^+$ and $(92n + Na)^+$ from the solvent (glycerol) background. Such conditions are reported (21) to imply that optimal sample preparation has not been

achieved because of either insufficient sample concentration or unsuitability of the solvent. Most likely, the relatively low carbon content of these samples is the reason that the spectra are difficult to interpret.

Anion Exchange HPLC

Ion-exchange chromatography is a flexible technique used mainly for the separation of ionic or easily ionizable species. Ion-exchange chromatography is carried out with packings that possess charge-bearing functional groups. The most common retention mechanism is simple ion exchange of sample ions and mobile phase ions with the charged groups of the stationary phase. This process involves competition between sample ions present in the mobile phase and the counter ions to pair with the oppositely charged fixed functional groups on the stationary phase. This means that the sample ions present in the mobile phase have to displace one or more of the counter ions that are paired with the fixed functional groups, in order to be adsorbed themselves (32). Sample retention in ion-exchange chromatography can also be controlled through variation of the mobile phase pH. Increasing the concentration of counterions in the mobile phase by either increasing the buffer concentration or by the addition of a neutral salt provides stronger competition between the sample and counterions for the exchangeable ionic centers and generally reduces retention. Sample

retention in anion exchange generally increases with increase in pH (33).

Figure 13 shows the chromatogram of Cross Lake sediment fulvic acid where acetic acid (pH 3.1) and acetic acid with triethylamine (pH 6.9) were used as the carrier solvents in a stepwise mode on a weak anion exchange (WAX) column. The chromatogram shows that two of the fractions were resolved in the acidic solvent (acetic acid) and one fraction was resolved in the solvent mixture (acetic acid with triethylamine). Weak acids of increasing Ka showed increasing retention on this column. The chromatogram indicates at least three types of weak acidic functionalities are presented in the sample. A recent publication by Ephraim et al. (34) identified four acidic functionalities in purified fulvic acid with pKa 1.8, 3.2, 4.2 and 5.7, respectively.

For equal concentrations of different mobile-phase ions in ion exchange, the retention of a given sample is generally increase in the sequence: citrate < oxalate < formate < acetate < OH (33). The three fractions I, II and III were collected by repeated injection of the aquatic solution of Cross Lake sediment fulvic acid. Each fraction was concentrated to the original volume and reinjected on reversed-phase (Partisil-10 ODS) column under isocratic mode using Solv.II as carrier solvent. Chromatograms in Figure

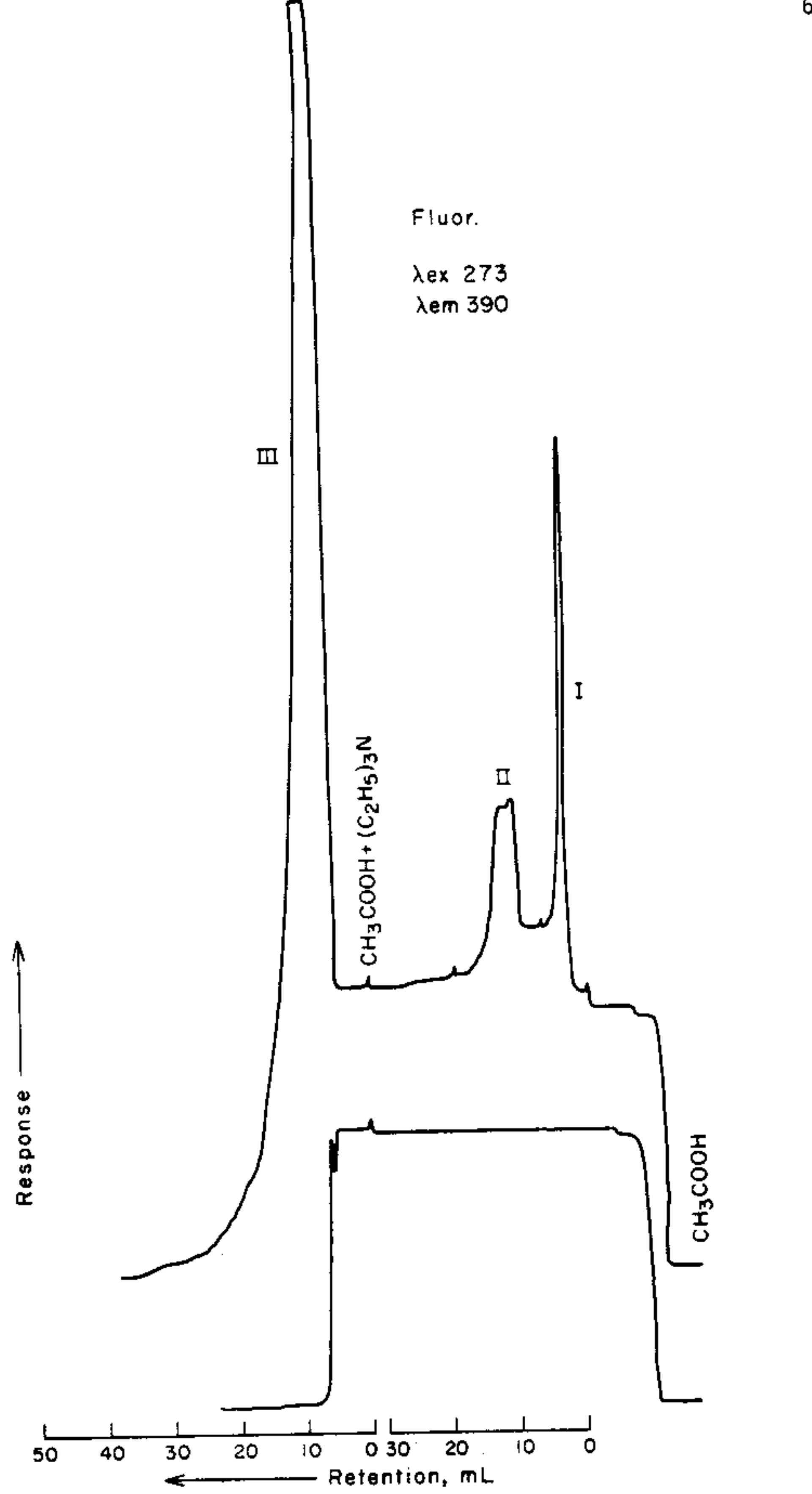

Figure 13--Chromatogram of Cross Lake sediment fulvic acid fractionated into three fractions by WAX column.

14 shows that each fraction is further resolved into 5-9 fractions. These results demonstrate the progressive fractionation of fulvic acid components by HPLC.

Reversed-Phase HPLC

Reversed-phase HPLC employing chemically bonded hydrocarbonaceous stationary phases has enjoyed widespread success primarily because of the large number of variable factors that can be adjusted in the polar mobile phase to give improved chromatographic performance. These factors include pH, ionic strength, polarity, dielectric constant, hydrogen bonding capability and concentration of surface-active ions (35, 36, 37). In reversed-phase HPLC, the driving force for solute retention is not the favorable affinity of the solute for the stationary phase, but rather the effect of the solvent forcing the solute to the hydrocarbonaceous layer of stationary phase (2).

Retention in reversed-phase HPLC is a function of sample hydrophobicity whereas the selectivity of the separation results almost entirely from specific interactions of the solute with the mobile phase. Generally, the selectivity may be conveniently adjusted by changing the type of organic modifier in the mobile phase. For ionic or ionizable solutes, pH buffers, which suppress ionization, or ion-pairing reagents, used to form lipophilic complexes, increase the degree of solute transfer to the stationary phase and may be used to control selectivity.

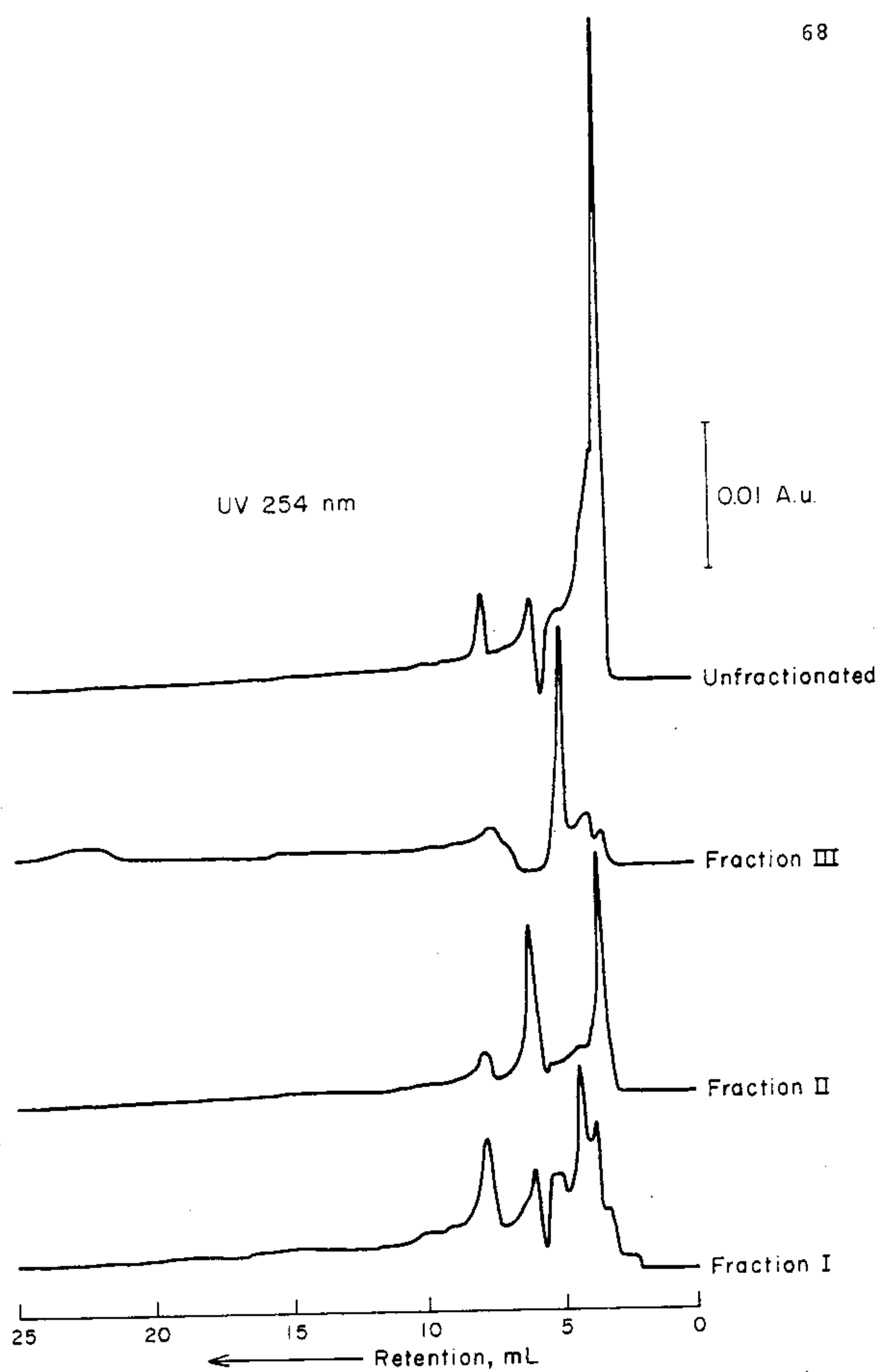

Figure 14--Chromatogram of Cross Lake sediment fulvic acid and its fractions (Figure 13); column, Partisil-10 ODS; solvent, Solv. II.

Through the selection of appropriate bonded phase and carrier solvent system RP-HPLC is ideally suited for separation of organic compounds of a wide range of polarity. Several studies have investigated the importance of various solute properties on retention in bonded phase reversed-phase chromatography. Recently Sadek et al. (38) showed that by use of the solvatochromic comparison method, the most important solute parameters effecting solute retention, are the solute size and hydrogen bond acceptor strength. The solute dipolarity is a minor but still significant factor.

The details of the mechanism governing retention in reversed-phase chromatography using chemically-bonded hydrocarbonaceous phase is not completely understood (2). However, the solvophobic theory provides a semiquantitative explanation of solute retention. Solute retention in reversed-phase chromatography could proceed either via partitioning between the hydrocarbonaceous surface layer of the nonpolar stationary phase and the mobile phase or by adsorption of the solute to the nonpolar portion of the stationary phase. The partitioning mechanism seem unlikely since the hydrocarbonaceous layer is only a monolayer thick and lacks the favorable properties of a bulk liquid for solubilizing the solutes. The evidence favors the adsorption mechanism either with the stationary phase

surface itself or by interaction with ordered solvent molecule layers at the stationary phase surface (2). The solvophobic theory assumes that aqueous mobile phases are highly structured due to the tendency of water molecules to self-associate by hydrogen bonding and that this structuring is perturbed by the presence of nonpolar solute molecules. As a consequence of the very high cohesive energy of the solvent, the less polar solute are literally "squeezed out" of the mobile phase and are bound to the hydrocarbon portion of the stationary phase. In this instance, the driving force for solute retention is not the familiar mechanism used to explain retention in other chromatographic system, the favorable affinity of the solute for the stationary phase, but rather the effect of the solvent forcing the solute to the hydrocarbonaceous layer. If the solute contains polar groups then the dipolar of hydrogen bonding interaction of these groups with the mobile phase will oppose the solute transfer mechanism.

Carrier Solvent Systems

In this research seven pairs of carrier solvent systems were used under isocratic and stepwise condition. These solvent systems included polar and less polar solvent in the presence or absence of acetic acid and ion pairing or organic modifiers. Several possible intermolecular interactions including hydrogen bonding, proton donor,

proton acceptor and dipole moments control the strength of these solvents. Methanol is a good proton acceptor and donor and it interacts preferentially with hydroxylated molecules (e.g., acids, phenols) as well as with basic samples (e.g., amines, sulfoxides). Acetonitrile tends to interact preferentially with sample molecules having large dipole moments (e.g., nitro-compounds, nitrile, amines). The acetic acid in Solv. A → B and Solv. C → D systems enhances the protonation effect. In Solv. SDS-A SDS-B and Solv. PICA PICA-M system, through adding the cationic and anionic pairing reagent, the predominant force between the molecules is dielectric interaction.

Tables VII and VIII show the pH and polarity of these carrier solvent systems. Table IX shows the capacity factor of two model compounds with each carrier solvent system. It is noted that, at low pH (solvent A, 3.43), the vanillic acid is almost completely in protonated, uncharged form and has a relatively high affinity for stationary phase and a relatively low affinity for the mobile phase; this causes the vanillic acid to move slowly through the reversed-phase column. At high pH (solvent A1, 6.61), vanillic acid exists almost completely in the unprotonated, negatively charged form, the carboxylate group is less adsorbophilic and more eluophilic which causes the charged molecule to move more rapidly through the column (39).

TABLE VII

pH AND POLARITY OF REVERSED-PHASE SOLVENT SYSTEMS

Solvent	pH	Polarity*	HPLC Condition
Solv. II	3.05	9.995	Isocratic
A	3.43	10.145	Stepwise gradient
B	3.96	5.861	A→B
A1	6.61	10.149	Stepwise gradient
B1	6.75	5.865	A1→B1
C	3.01	10.152	Stepwise gradient
D	4.00	6.456	C→D

* Polarity
$P' = \Phi a\ Pa + \Phi b\ Pb$

Φa, Φb : volume fraction of solvent A and solvent B

Pa, Pb : P' (solvent polarity parameter) of pure solvent A and B.

TABLE VIII

pH AND POLARITY OF ION-PAIR SOLVENT SYSTEMS

Solvent	pH	Polarity*	HPLC Condition
Octylamine	6.34	9.540	Isocratic
SDS-A	2.64	10.199	Stepwise gradient
SDS-B	2.67	7.777	SDS-A ⟶ SDS-B
PICA	6.99	10.200	Stepwise gradient
PICA-M	7.67	8.925	PICA ⟶ PICA-M

* same as described in TABLE VII.

TABLE IX

THE CAPACITY FACTOR, k', OF MODEL COMPOUND WITH FIVE CARRIER SOLVENT SYSTEMS

Column: Partisil-10 ODS

kR': Capacity Factor of Resorcinol

kV': Capacity Factor of Vanillic Acid

Solvent System	kR'	kV'
Solv. A	1.10	8.55
Solv. C	0.33	3.66
Solv. Al	1.5	0.5
Solv. SDS-A	1.5	7.5
Solv. PICA	1.33	0.66

All the capacity factor k' were measured in the polar solvent only.

Solvent pair A1 → B1 was selected for further studies on fulvic acid and model compounds. The retention behavior of fourteen compounds were determined by using Nova Pak column and Solv. A1 → B1 as the carrier solvent. Table X shows the k' of the model compounds. It is noted that all the selected amino acid compounds, pyridine, and o-cresol were eluted in the less polar solvent (B1). This may suggest that these or similar moieties which occur in the fulvic acid are fractionated in the less polar solvent (B1).

<u>Optimization of Fluorescence and U.V. Detection</u>

To maximize the utilization of the detectors, selected samples were run at three u.v. absorption wavelengths and five combinations of fluorescence excitation and emission wavelengths. The u.v. wavelengths were 214 nm, 254 nm, 313 nm and the excitation/emission wavelengths chosen were 273 nm/390 nm, 350 nm/427 nm, 350 nm/485 nm, 385 nm/420 nm, 385 nm/475 nm. Figure 15 shows that a fluorescence chromatogram of aquatic fulvic acid at the five selected wavelengths by using Partisil-10 ODS column and Solv. II as the carrier solvent. The general configuration of these chromatograms are comparable but differ in intensity. Three characteristic responses with k' 0.40, 1.10, 1.61 and α 2.75, 1.46 are noted at λex 273 nm, λem 390 nm, and λex 350 nm, λem 427 nm. The third peak was not resolved at the other wavelengths. The u.v. chromatograms at the three

TABLE X

CAPACITY FACTOR OF MODEL COMPOUNDS

Column: Nova Pak   Solvent: Solv. A1 → B1

Compound	Capacity Factor (k') in A1
Resorcinol	3.92
Vanillic acid	0.96
Adipic acid	1.40
Alanine	*
2,4-Dinitrophenol	2.35
Proline	*
Aspartic acid	*
Salicylic acid	0.23
Furoic acid	0.17
3,5-Dinitrosalicylic acid	3.08
Serine	*
Pyridine	*
o-Cresol	*
Ethylenediamine	?

* peak show up only at the Solv. B1

? uncertain

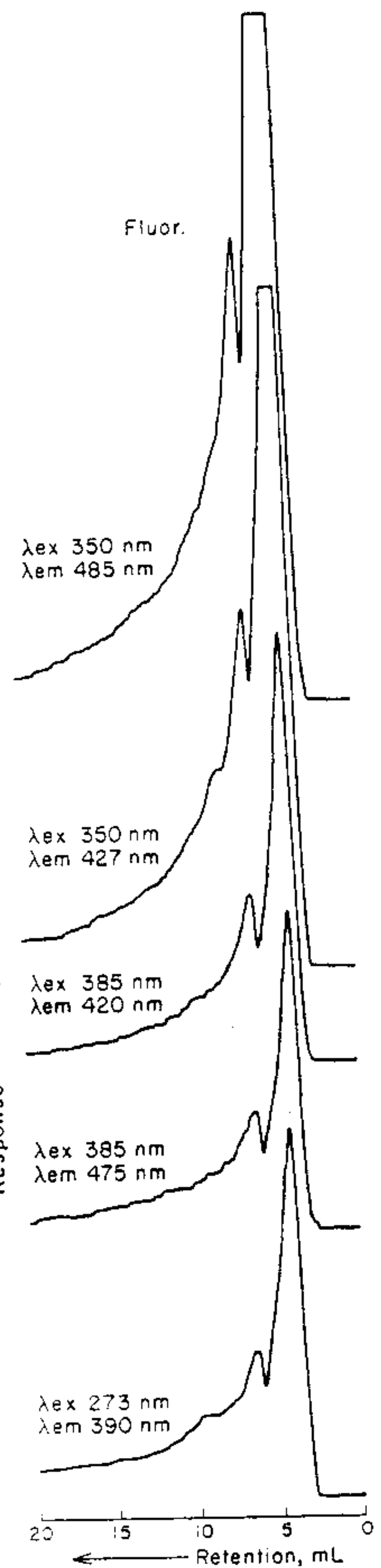

Figure 15--Five combinations of fluorescence excitation and emission wavelengths. Sample, Cross Lake sediment FA; eluent, Solv. II; column, Partisil-10 ODS; flow rate 1 mL/min; condition, isocratic.

selected wavelengths were comparable in configuration and increased in intensity in the order 214 nm > 254 nm > 313 nm. UV absorption at 254 nm is commonly used in HPLC to detect aromatic rings and conjugated structures (40). Figure 16 shows a fluorescence chromatogram of aquatic sediment fulvic acid at three selected wavelengths under stepwise gradient condition. Figure 17 shows the fluorescence chromatogram of aquatic water fulvic acid. Table XI shows the k' for chromatogram in Figures 16 and 17. Some discrepancy is noted in k' of SDS-A but excellent reproducibility exists in k' of SDS-B. Further experiments described in this research were conducted at λ ex 273 nm, λ em 390 nm and u.v. 254nm.

Comparison between C_{18} and C_8 Columns

Table XII shows the comparison of the capacity factors of seven model compounds and Cross Lake sediment fulvic acid as well as Ogeechee River fulvic acid on Partisil-10 ODS and Rsil phenyl columns. From this table we find that the capacity factor of these two column were comparable. However, the resolution and stability of Partisil column was better than the Rsil phenyl column. Octadecylsilica columns are often favored because of their higher stability when exposed to water-rich eluents; this stability is attributable to the protection of the silica matrix by the long alkyl chains (41).

Fluor.

λex 380 nm
λem 427 nm

λex 273 nm
λem 420 nm

λex 273 nm
λem 390 nm

SDS-B

SDS-A

Response

60 40 20 0 40 20 0

Retention, mL

Figure 16--Three combinations of fluorescence excitation and emission wavelengths. Sample, Lake Pat Mayse sediment FA; eluent, SDS-A and SDS-B; column, Partisil-10 ODS; flow rate, 2 mL/min; condition, stepwise gradient.

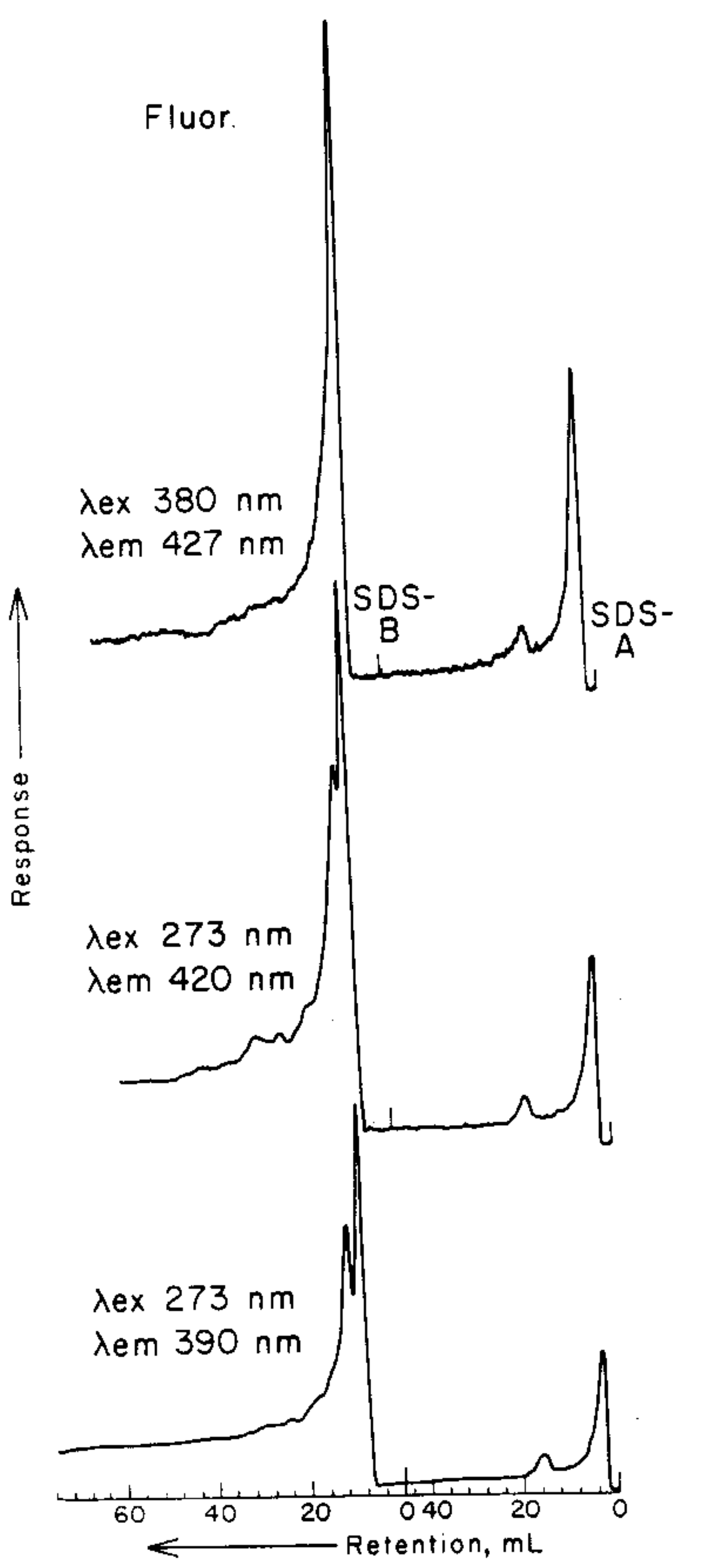

Figure 17--Three combinations of fluorescence excitation and emission wavelengths. Sample, Lake Pat Mayse water FA; the operating conditions same as described in Figure 16.

TABLE XI

CAPACITY FACTOR, k', OF LAKE PAT MAYSE SEDIMENT AND WATER FULVIC ACID AT DIFFERENT FLUORESCENCE EXCITATION AND EMISSION WAVELENGTHS

Sediment Fulvic Acid		
Wavelength	Capacity Factor in SDS-A	Capacity Factor in SDS-B
ex 273, em 390	0.15, 2.18, 4.10	1.48, 2.44, 6.58, 8.36, 12.38
ex 273, em 420	0.15, 4.73	1.48, 2.44, 6.58, 8.36
ex 350, em 427	0.21, 1.68, 3.27	1.48
Water Fulvic Acid		
ex 273, em 390	0.02, 2.44	1.48, 2.44, 6.58, 8.36
ex 273, em 420	0.02, 4.99	1.48, 2.44, 6.58, 8.36
ex 350, em 427	0.02, 5.11	1.48

TABLE XII

COMPARISON BETWEEN CAPACITY FACTOR, k', OF MODEL COMPOUNDS AND TWO FULVIC ACIDS ON C_{18} AND C_8 COLUMNS

	k' in Solv. A Partisil-10 ODS	k' in Solv. A Rsil phenyl
Phenol	2.44	3.96
Resorcinol	1.10	1.29
3-Methylcatechol	*	*
4-Methylcatechol	2.95	2.31
Vanillic acid	8.55	8.23
2,4-Dihydroxybenzoic acid	4.54	2.82
2,4,6-Trihydroxybenzoic acid	3.33	2.84
Ogeechee FA	0.78, 3.96	0.27, 4.09
Cross Lake FA	0.53, 3.84	0.59, 3.65

* peak show up only at Solv. B

Comparison between Two Carrier Solvent Systems (A → B) and (C → D)

Solvent selectivity is controlled by the selectivity group reflecting contributions from donor. acceptor, and dipole characteristics of the solvent, respectively (42, 43). Met..anol is a good proton acceptor and donor and it interacts preferentially with hydroxylated molecules (e.g., acids, phenols) as well as with basic samples (e.g., amines, sulfoxides). Acetonitrile tends to interact preferentially with sample molecules having large dipole moments (e.g., nitro-compounds, nitrile, amines). The polarity and pH for these two solvents are similar. Figures 18,19 show the u.v. and fluorescence chromatograms of fulvic acid using two carrier solvent systems. The figure indicates that each solvent system has essentially the same total area of response and that the u.v. absorbing component of aquatic fulvic acid is almost equally distributed between the polar and less polar solvents. These results indicate that the intermolecular interactions (e.g., hydrogen bonding, dipole moments) operating on fulvic acid with these two solvents are essentially the same. It is also noted that solvent pair C → D gives better resolution than solvent pair A → B. But the high volatility of acetonitrile gives some operational difficulty for solvent pair C → D.

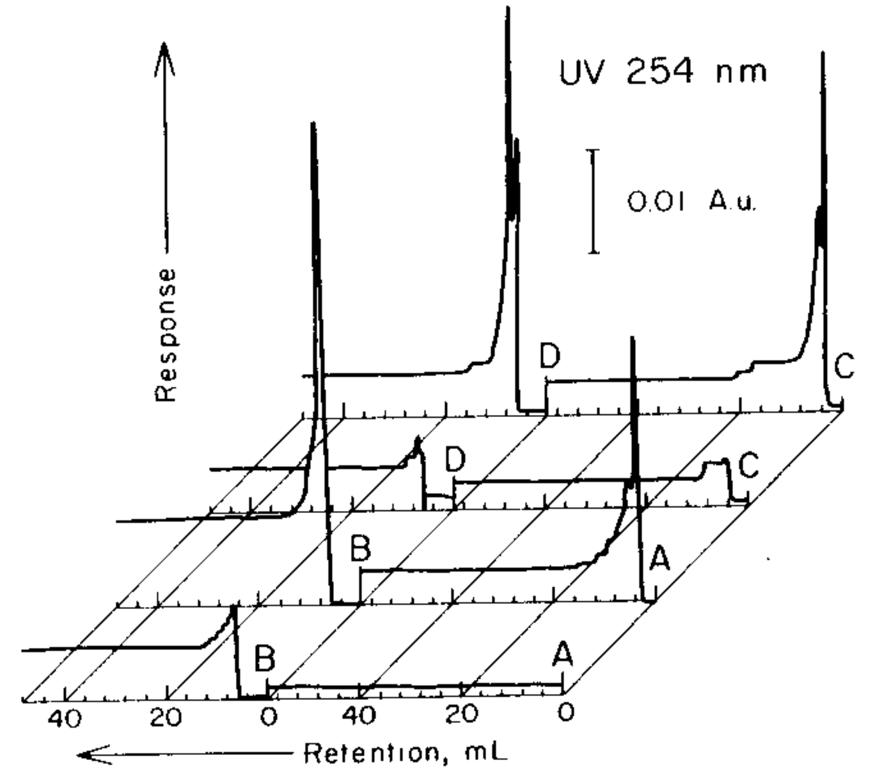

Figure 18--UV chromatogram of two solvent systems. Sample, Lake Pat Mayse sediment FA; eluent, Solv. A → B and Solv. C → D; the conditions same as described in Figure 16.

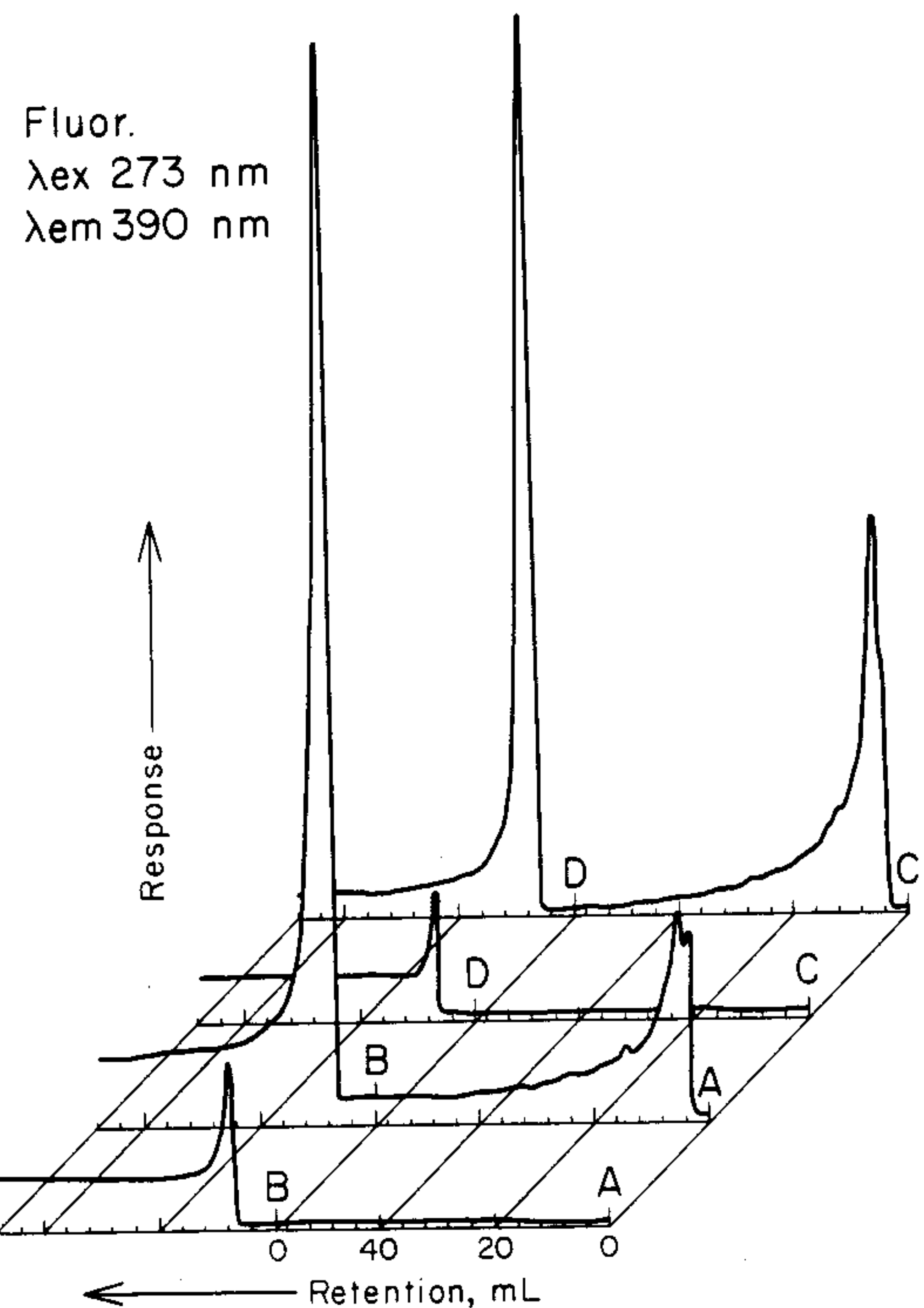

Figure 19--Fluorescence chromatogram of two solvent systems. Sample, eluent, and the operating conditions same as described in Figure 18.

pH of Carrier Solvent

Changes in pH can change the separation selectivity for ionized or ionizable solutes, since charged molecules are distributed preferentially into the aqueous or more polar phase (33). Crathorne et al. (44) pointed out that decreasing the pH decreased the degree of ionization of the solute and made it less polar. Figure 20 shows fluorescence and u.v. chromatograms of Lake Pat Mayse sediment and water FA with the carrier solvent A1 → B1. Response in the A1 or A region represents the unretained and/or the polar components of fulvic acid while response in the B1 and B regions represent the less polar components. Examination of Figures 18-20 show that about only 25-35% of the total u.v. and fluorescence responses are in the B1 solvent (without acetic acid, pH 6.61) and 50-60% of the total responses represent in the B less polar solvent (with acetic acid, pH 3.43). This can be related to secondary equilibrium and the influence of acetic acid on the carrier solvent, and the subsequent protonation of some of the fulvic acid components. These results present the first chromatographic evidence on the labile nature of some of fulvic acid components.

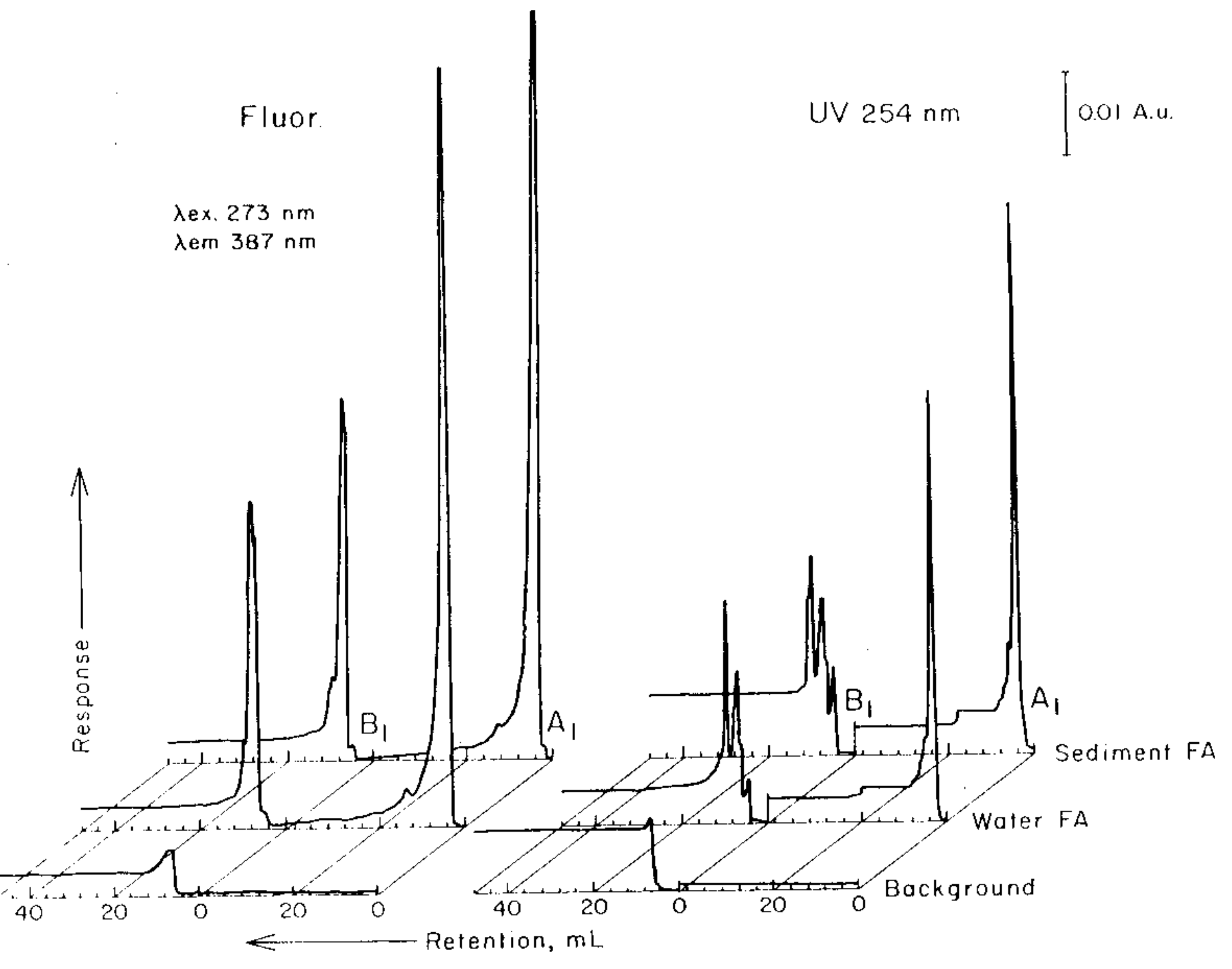

Figure 20--Chromatograms of Lake Pat Mayse sediment FA. Column, Nova Pak; eluent, Solv. A1 and B1; flow rate, 2 mL/min; condition, stepwise gradient.

pH of Sample

It is expected that sample pH would have little influence on the shape of the chromatogram. Figures 21 and 22 show chromatograms of water fulvic acid at pH 7.01 and 2.08 by using carrier solvent systems A → B and A1 → B1. It is noted that sample pH has little effect on the general configuration of the u.v. and fluorescence chromatograms. As expected a change in sample pH has less pronounced effect on the general configuration of the HPLC chromatogram than the carrier solvent pH.

Comparison of Water and Sediment Fulvic Acids from Different Locations

Figure 20 shows the fluorescence and u.v. chromatograms of Lake Pat Mayse water fulvic acid and sediment fulvic acid by solvent pair A1 → B1. Both water and sediment fulvic acids were fractionated into the same major components. At least five u.v. and fluorescing components were fractionated in solvent B1 (85% Methanol-Water) in both water and sediment samples. All water and sediment samples analyzed under these conditions showed chromatograms of the same general configuration. Figures 23-26 show the comparison of sediment and water fulvic acid from different locations by using solvent system C → D. As found in case of solvent A1 → B1, the chromatograms show that sediments from all locations as well as water fulvic acids were fractionated

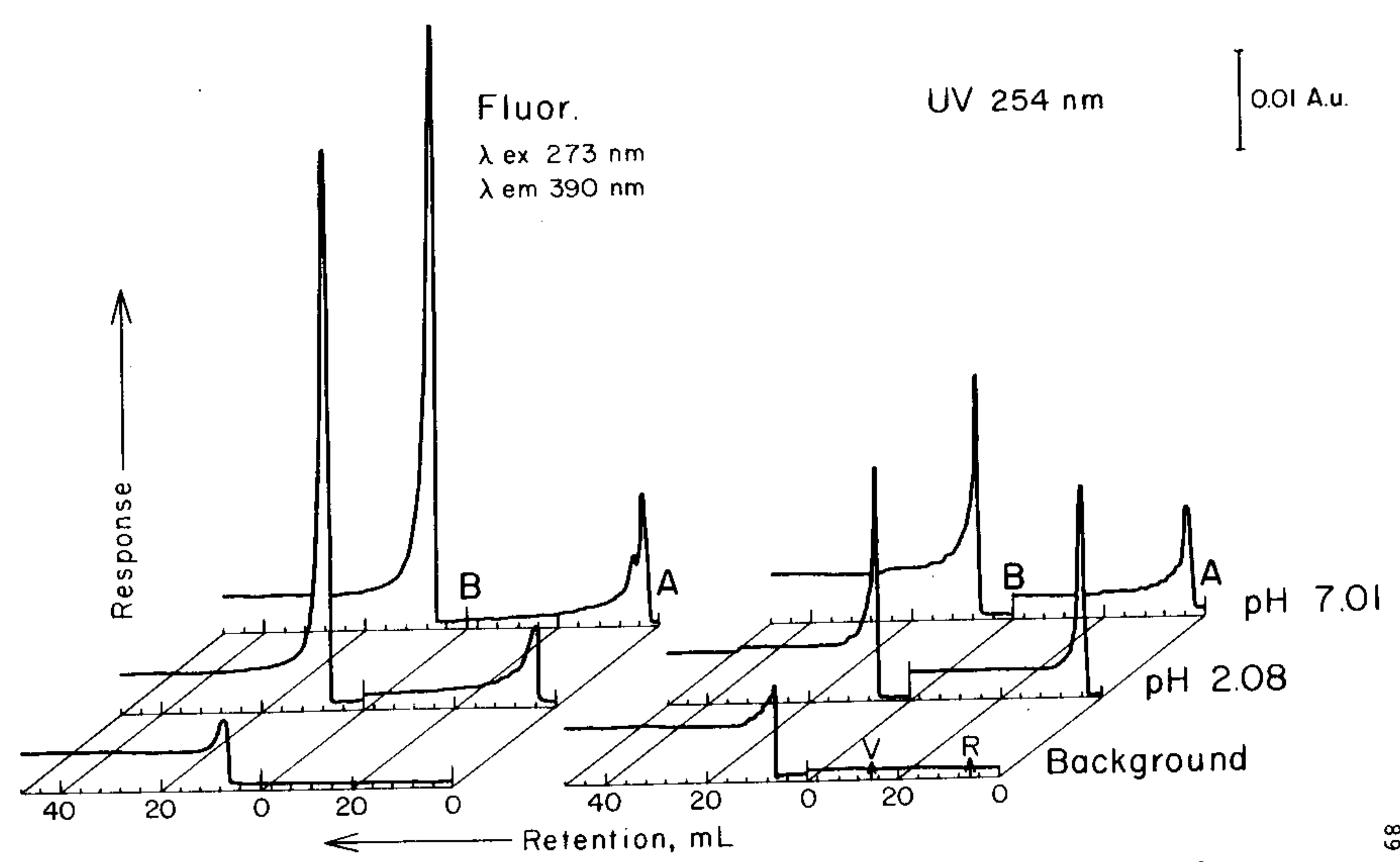

Figure 21--Effect of sample pH. Sample, Lake Pat Mayse water FA; eluent, A and B; the operating conditions same as described in Figure 16.

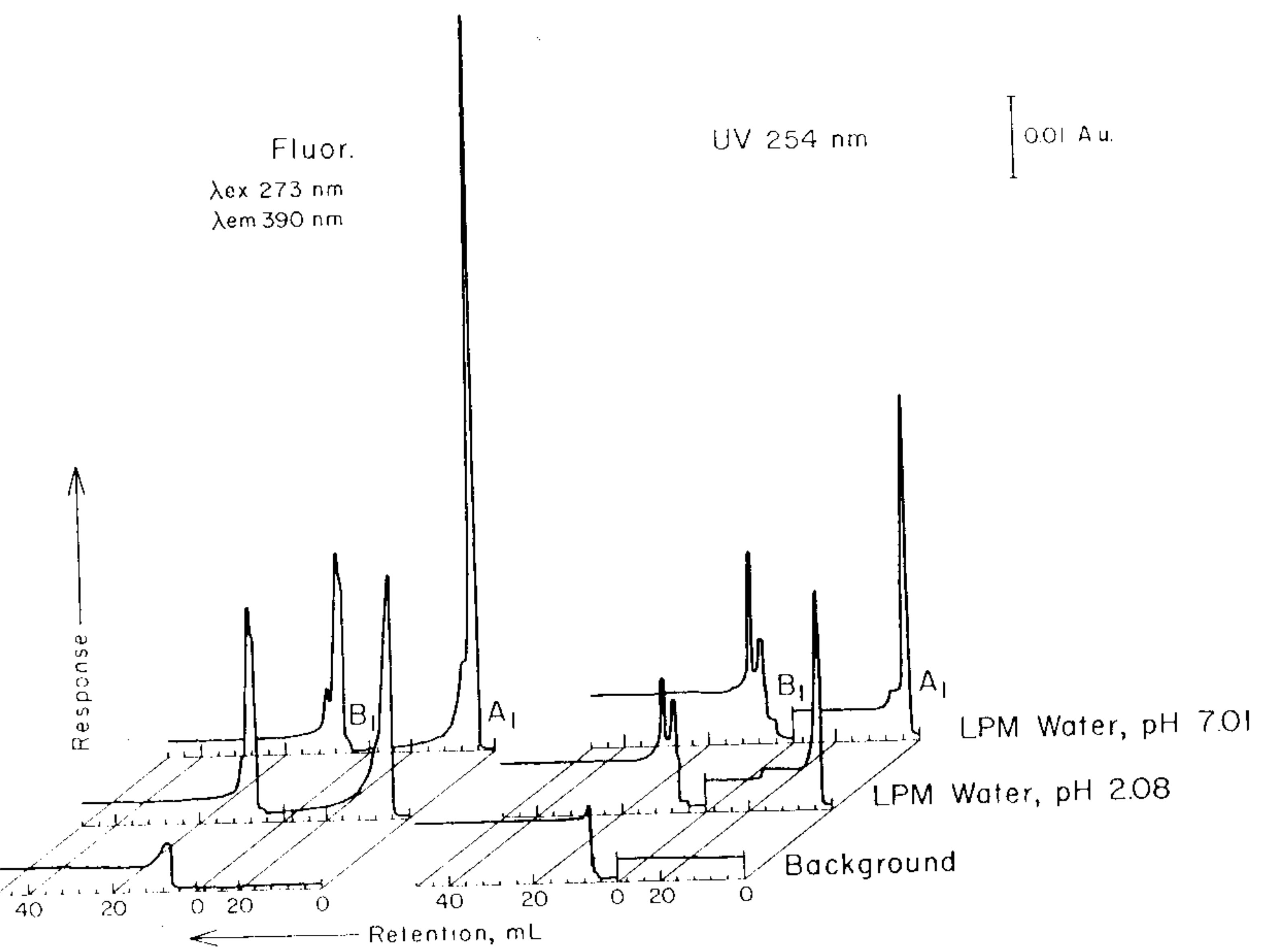

Figure 22--Effect of sample pH. Sample, Lake Pat Mayse water FA; eluent, Solv. A1 and B1; the conditions same as described in Figure 16.

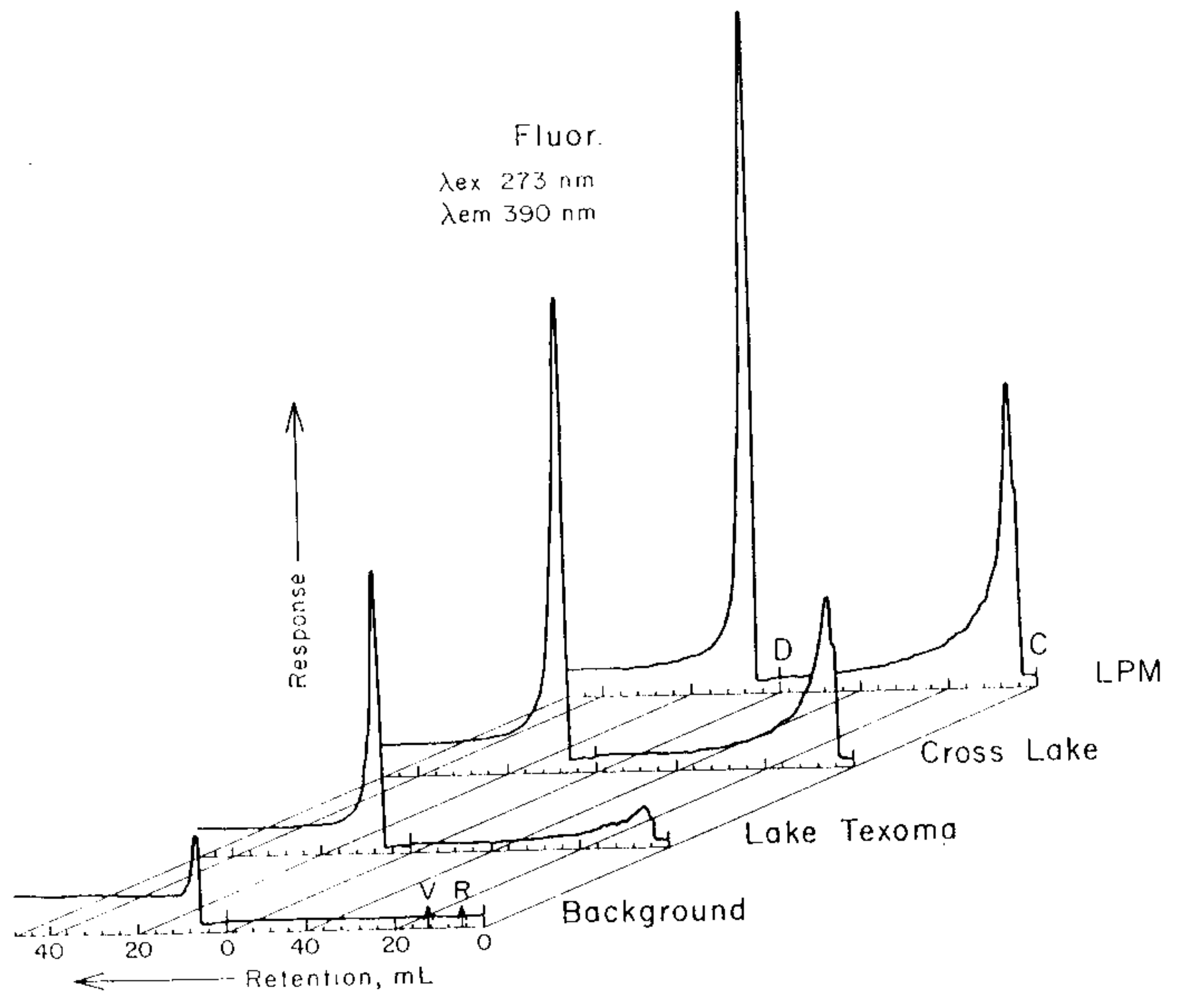

Figure 23--Fluorescence chromatogram of three sediment FA. Sample, Lake Pat Mayse, Cross Lake and Lake Texoma sediment FA; eluent, Solv. C and Solv. D; the operating conditions same as described in Figure 16.

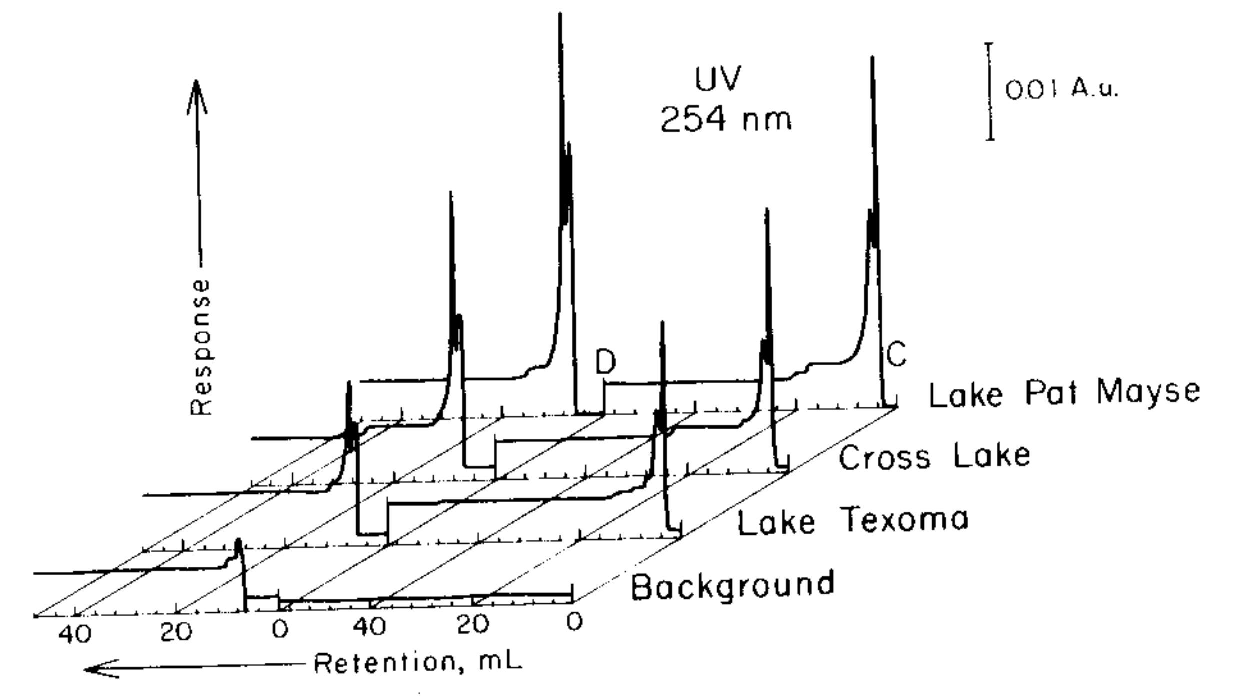

Figure 24--UV chromatogram of three sediment FA. Sample and conditions same as described in Figure 23.

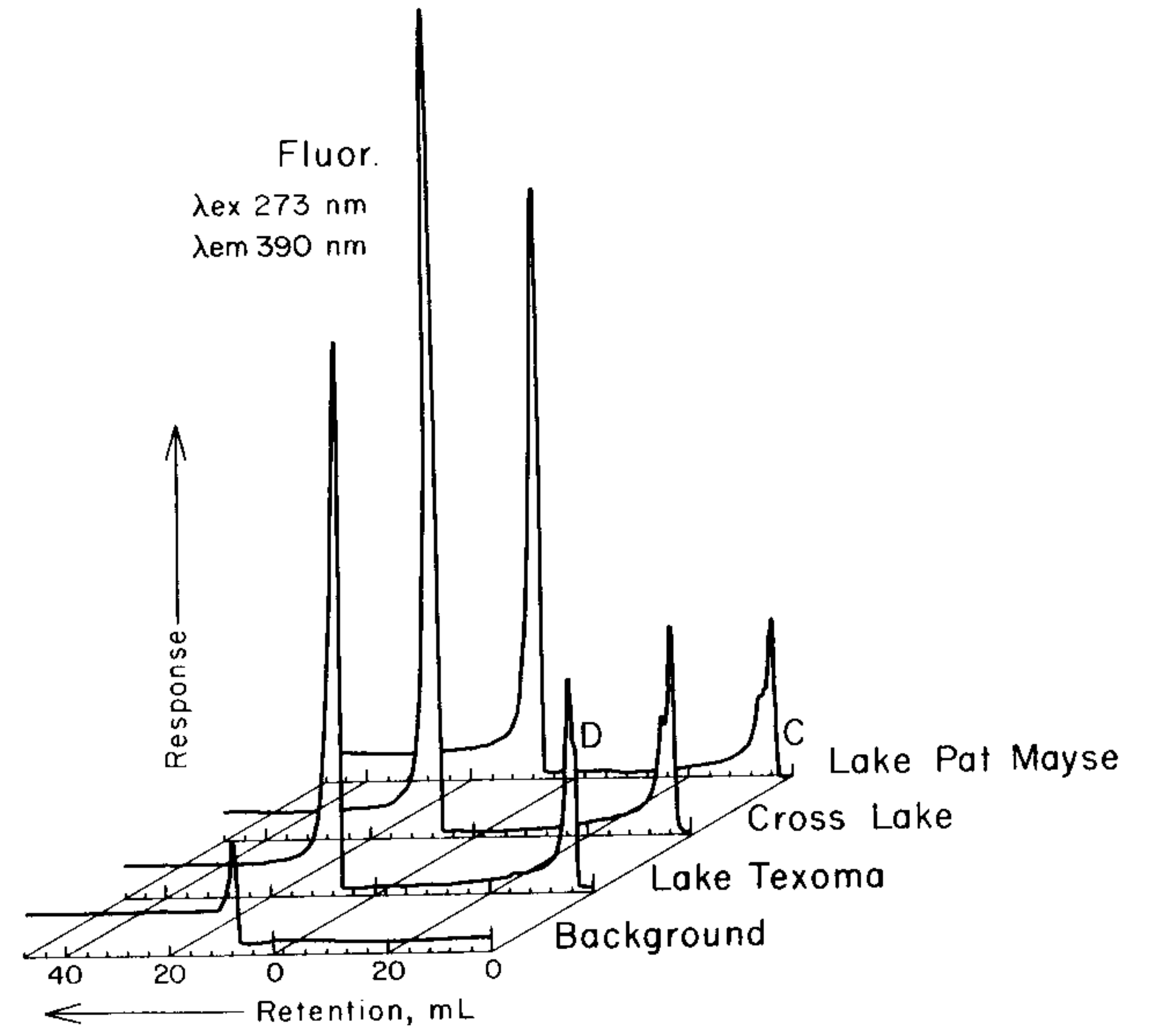

Figure 25--Fluorescence chromatogram of three water FA. Sample, Lake Pat Mayse, Cross Lake and Lake Texoma water FA; the conditions same as described in Figure 23.

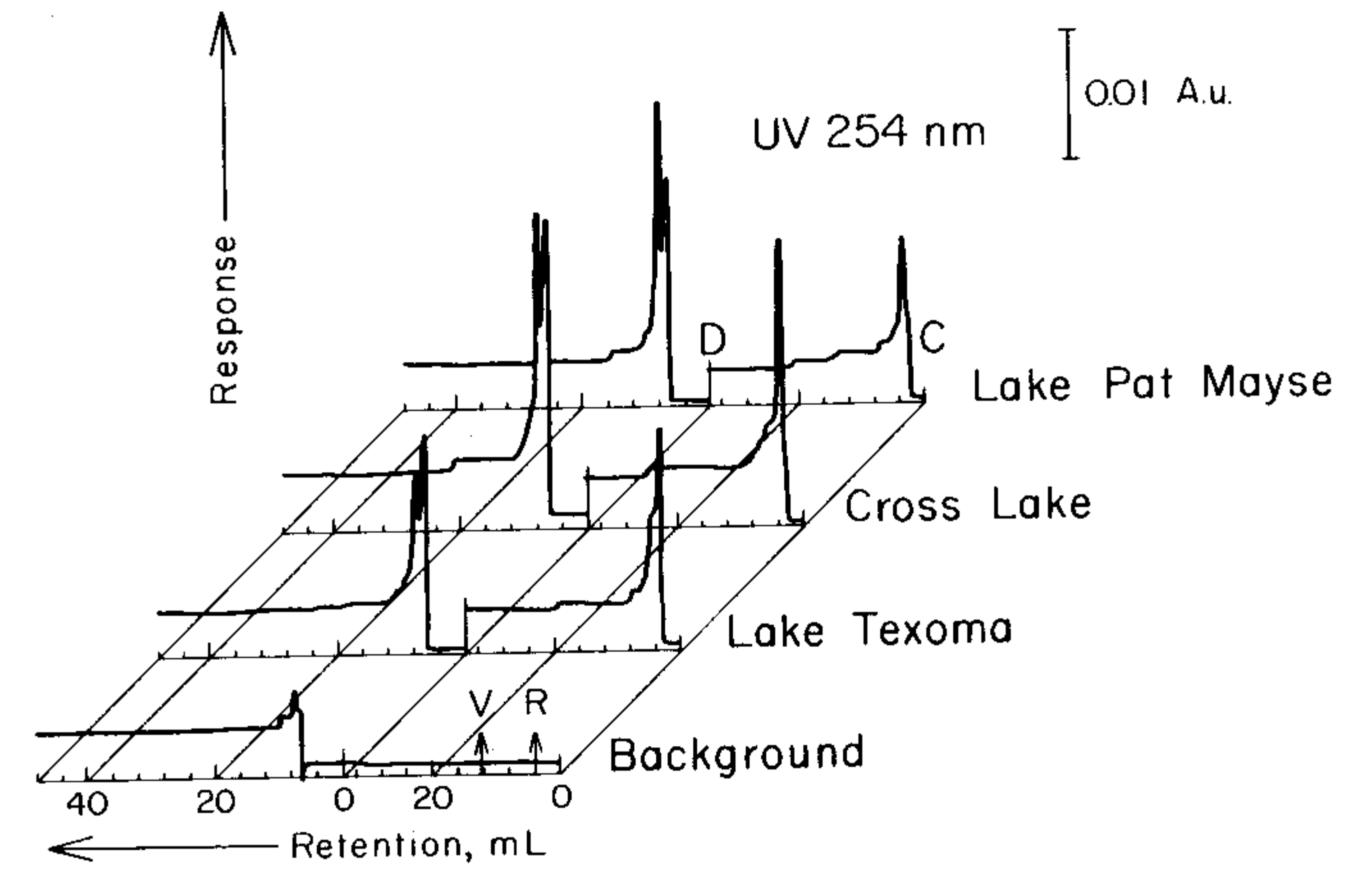

Figure 26--UV chromatogram of three water FA. Sample and the conditions same as described in Figure 25.

into the same two major components. Similar results were obtained by the other carrier solvent systems and reference fulvic acids. It was also found that fulvic acid extracted from water and sediment from the same site were more similar than those extracted from different sites (Figures 23-26).

Semipreparative Fractionation of Fulvic Acid

From the seven carrier solvent systems, A1 → B1 solvent system was selected to fractionate reference fulvic acid. The chromatograms indicated well defined separation of two major components of fulvic acid. In Solv. B1, the less polar components of fulvic acid seems to indicate a regular retention pattern which may correspond to a homologous series of structures. This solvent system presented reasonable resolution, stability and ease to operate during freeze-dried process. A total amount of 70 mg suwannee reference fulvic acid was fractionated into two main fractions by using ST/C_{18} semipreparative scale column and Solv. A1 → B1 as the carrier solvent under stepwise gradient condition. Fractionation was made in batches of 10 mg fulvic acid. Fractions of each batch were freeze-dried then desiccated to a constant weight. Separation efficiencies were checked by reinjecting aqueous solution of the fractions on the analytical column, with Solv. A1 and B1. In some cases chromatograms indicated further decomposition of the fractions. Only undecomposed fractions are kept for

further spectroscopic measurements. Table XIII shows the recovery amount of each fraction. The recovery of this fractionation higher than 100% can be attributed to the moisture and the release of the packing material.

Ion-Pairing Chromatography

A method for performing extractions of ionised solutes into organic phases has been studied for a number of decades. Ions of opposite electrical charge are added to the aqueous phases resulting in ion-pairing between the solute ion and pairing ion. The resultant complex has a low net electrical charge or polarity, is thus poorly hydrated, and so now can transfer readily to organic phases. The ion pairs are defined as Coulombic association species formed between two ions of opposite electrical charge. Their formation, however, is dependent upon many variables including ion constitution and polarisability and solvent dielectric. For chromatographic purposes ion pairs are formed between inorganic-organic and organic-organic solute pairs, and the formation of each type is highly dependent upon the immediate environment of both ions (35).

Essentially, two modes of reversed-phase ion-pair chromatography can be distinguished: (i) a gemini reversed-phase ion-pair system in which the pairing ion remains mainly in the mobile phase (in the absence of solute ions); (ii) and ion-pair exchange system in which the

TABLE XIII

RECOVERY OF SUWANNEE RIVER FULVIC ACID FRACTIONATION

Amount	Amount in A1	Amount in B1	Total Amount
10 mg	9.6 mg	5.3 mg	14.9 mg
10 mg	8.2 mg	5.0 mg	13.2 mg
10 mg	4.1 mg	4.3 mg	8.4 mg
10 mg	8.8 mg	7.1 mg	15.6 mg
10 mg	9.3 mg	5.2 mg	14.5 mg
10 mg	8.2 mg	5.6 mg	13.8 mg
10 mg	6.5 mg	5.9 mg	12.4 mg

pairing ions are already concentrated, mainly in the stationary phase as their own ion pair with their counter ion or gegenion. Retention of solutes then takes place by exchange with this counter ion. In reversed-phase ion-pair systems, the stationary phase can be either an organic liquid (ion-pair partition), or a hydrophobic surface (ion-pair adsorption). In both cases a hydrophobic packing is needed, in the first case in order to immobilize the organic liquid, in the second case in order to serve as an adsorbent (32).

Several models have been proposed for the actual mechanism of separation.

Pairing in the mobile phase model. A large counter-ion is added to the mobile phase, forming an ion pair with the ionized sample. This ion pair behaves as an electrically neutral, nonpolar compound, thus retaining on a reverse-phase column (45).

Ion exchange at surface model. Ion-exchange between solute and pairing reagent occurs at the surface of the packing. The organic part of the counter ion (PIC reagent) partition into the C layer of the column, exposing the ionic functionality to the mobile phase and the ionic compounds of interest; thus, the separation occurs due to an ion-exchange mechanism (45).

Ion interaction model. The ion interaction mechanism assumes dynamic equilibrium of the lipophilic ion resulting in the formation of an electrical double layer on the packing surface. Sample retention results from an electrostatic force caused by surface charge density and from an additional "sorption" of the lipophilic portion of the sample molecule onto the nonpolar surface (45).

Tetrabutylammonium ion (PICA) is an anionic paired reagent. Alkylammonium ions have the property of being aprotic and may therefore be used at all pH values; since they are both hydrophobic and ionised, they are able to form water structure enforced ion pairs in environments having a high dielectric constant, although this tendency will be reduced the shorter the alkyl chain is (35).

Figure 27 shows the fluorescence chromatogram of Lake Texoma sediment fulvic acid by using PIC-A carrier solvent system under isocratic condition. The upper and lower chromatograms are duplicate injections of the fulvic acid. It is noted that the fulvic acid is resolved into 8-9 peaks which give the best resolution in all solvents under isocratic condition. The value of k' ranges from 1.80 to 12. This can be related to the tetrabutylammonium ion form an ion pair with fulvic acid. This ion pair behaves as an electrically neutral, nonpolar compound, thus retaining on the column. However, inherent the problem was encountered

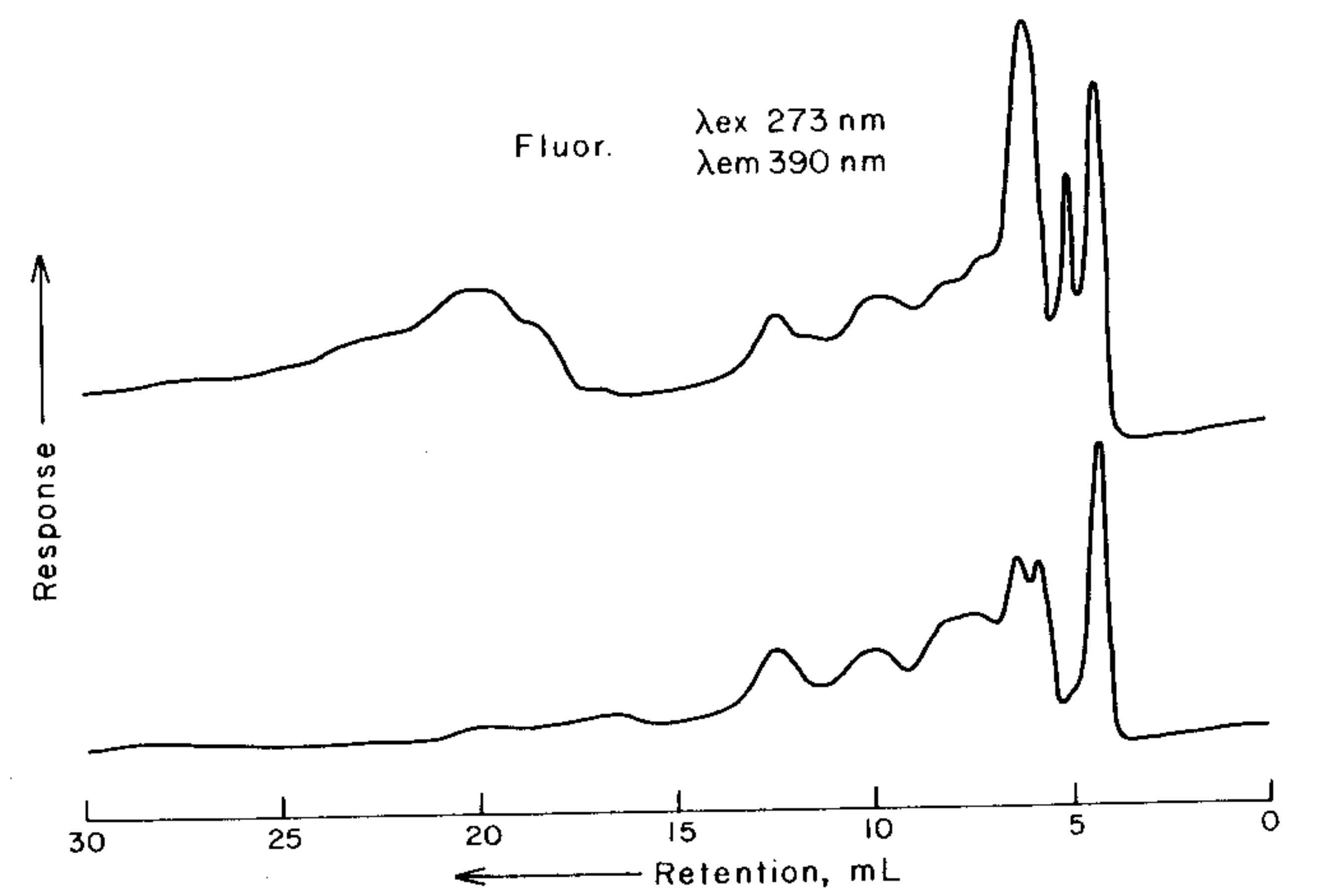

Figure 27--Fluorescence chromatogram of FA with ion-pairing reagent. Sample, Lake Texoma sediment FA; eluent, PICA; the operating conditions same as described in Figure 15.

in the reproducibility even after adding a precolumn. Figures 28 and 29 show the fluorescence and u.v. chromatograms of Lake Pat Mayse and Ogeechee River fulvic acid by using PICA and PICA-M carrier solvent system under stepwise gradient condition. The u.v. and fluorescence response were not any better than any of the other carrier solvent systems.

Soap Chromatography

This separation is based on a reversed-phase in combination with hydrophilic eluent containing methanol as an organic modifier and small concentration of a detergent (e.g., sodium 1-dodecyl sulfate, SDS) which forms an ion-pair with an ionized form of a solute (46, 47). The detergent is adsorbed by the reversed-phase surface to form a layer which is in some ways akin to an ion exchanger. The retention is due to the interaction between the neutral pair and nonpolar stationary phase. To keep the solutes in the preferred ionic forms, the pH of the carrier solvent is controlled. Speed of separation, selectivity and resolution can readily be adjusted by altering any one of a number of parameters such as the nature and concentration of the organic modifier in the eluent, the nature and concentration of the detergent, the nature and concentration of the acid, and by addition of salts, particularly salts with coordinating metal anions.

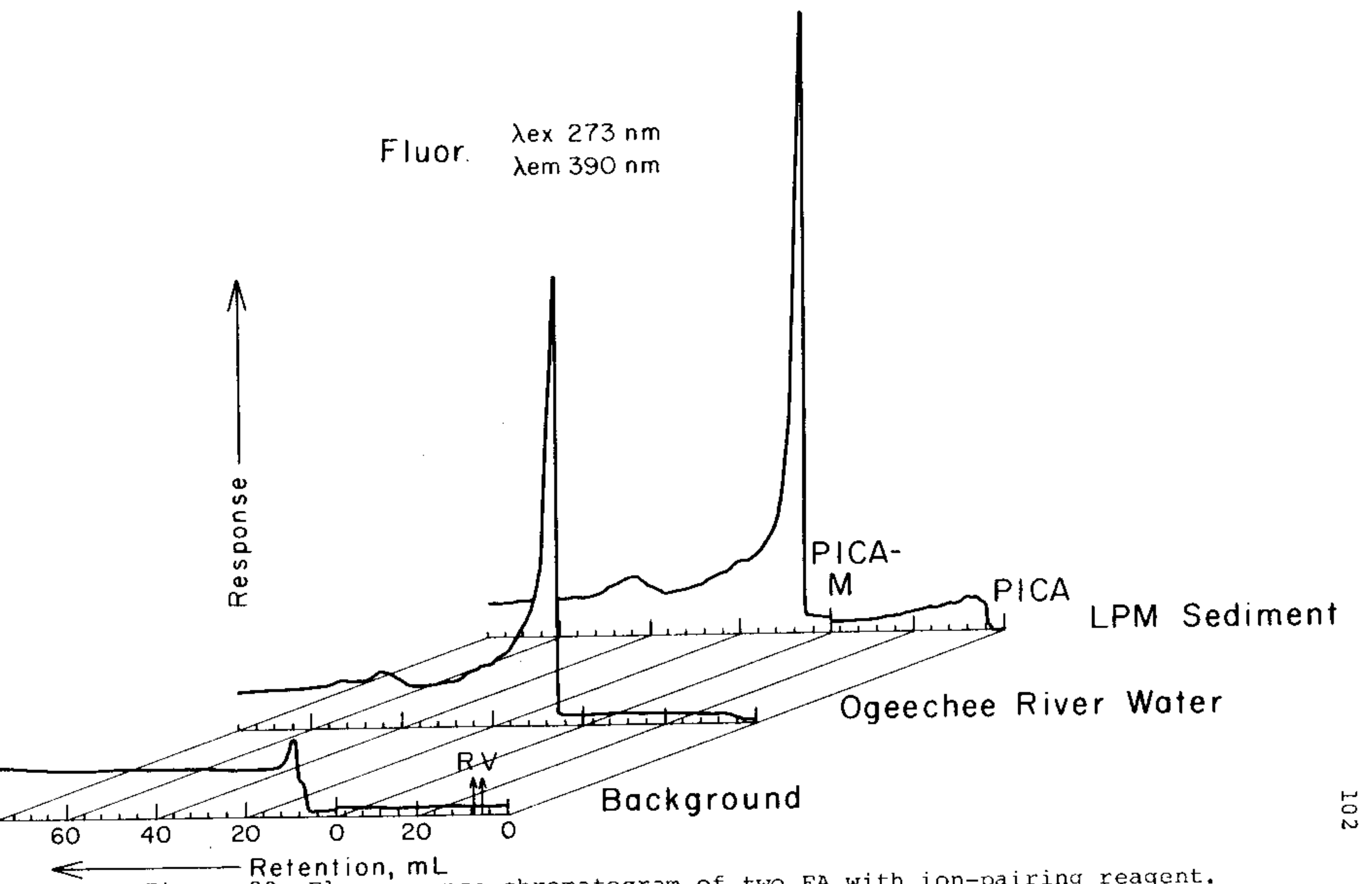

Figure 28--Fluorescence chromatogram of two FA with ion-pairing reagent. Sample, Lake Pat Mayse sediment and Ogeechee River water FA; eluent, PICA and PICA-M; the operating conditions same as described in Figure 16.

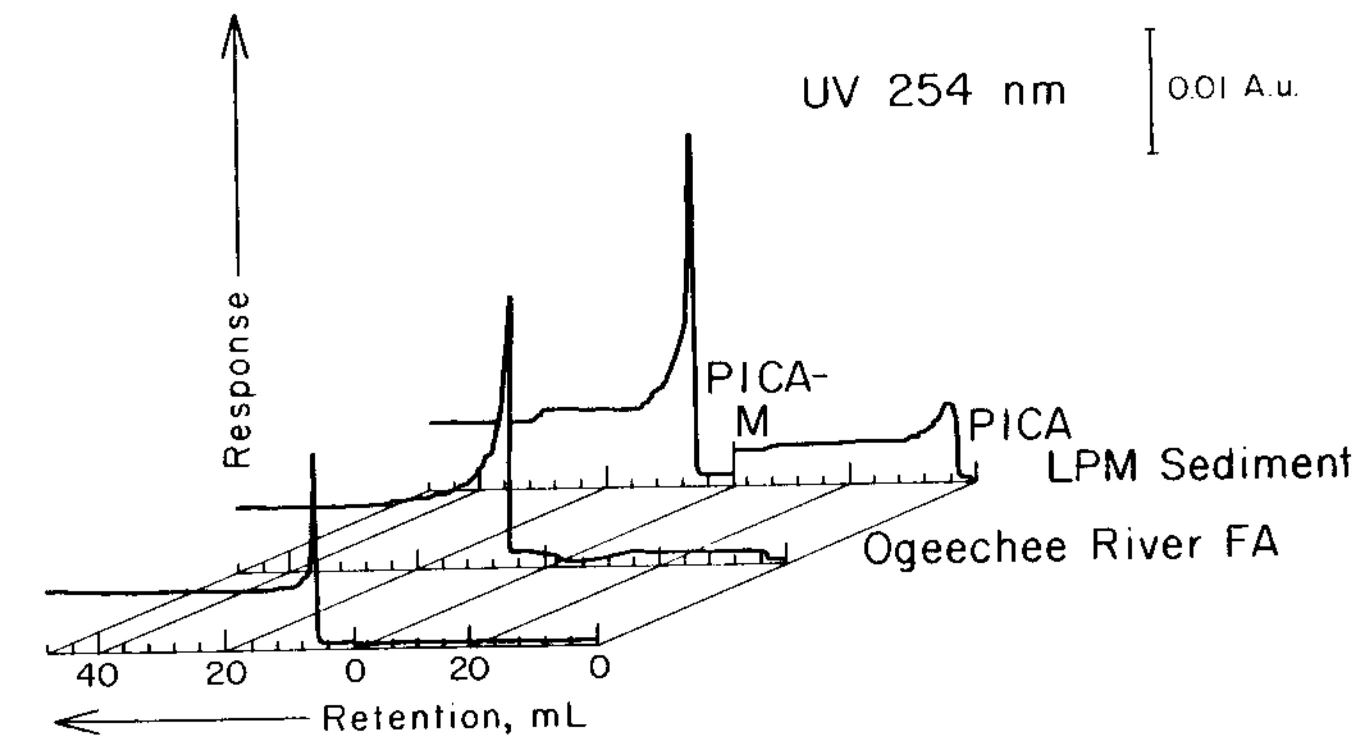

Figure 29--UV chromatogram of two FA with ion-pairing reagent. Sample and the conditions same as described in Figure 28.

Figures 30, 31 show the comparison of sediment fulvic acid from different locations by using solvent system SDS-A and SDS-B. Figures 32, 33 show the comparison of Pat Mayse sediment fulvic acid and water fulvic acid as well as the reference fulvic acid from Ogeechee river by the same solvent system. The chromatogram shows that sediments from all locations as well as water fulvic acids were fractionated into the same two major components. It is noted that this solvent pair provided the best fluorophore resolution. The decreasing of the u.v. and fluorescence response in the freeze-dry sample (water fulvic acid) can be attributed to the volatilization of some of the polar low molecular weight compounds during the freeze-drying procedure.

Ion-Interaction Chromatography

Ion interaction is taken to mean that any process in which ions interact because of Coulombic and other forces. These forces are referred to as electrostatic, eluophilic (having an affinity for the mobile phase), eluophobic (having an aversion for the mobile phase), adsorbophilic (having an affinity for the stationary phase), and adsorbophobic. The adsorbophilic ion that is intentionally added to the mobile phase are referred to as the "ion-interaction reagent" (48). Bidlingmeyer et al. (48) have suggested that neither the "ion-pair" model nor the

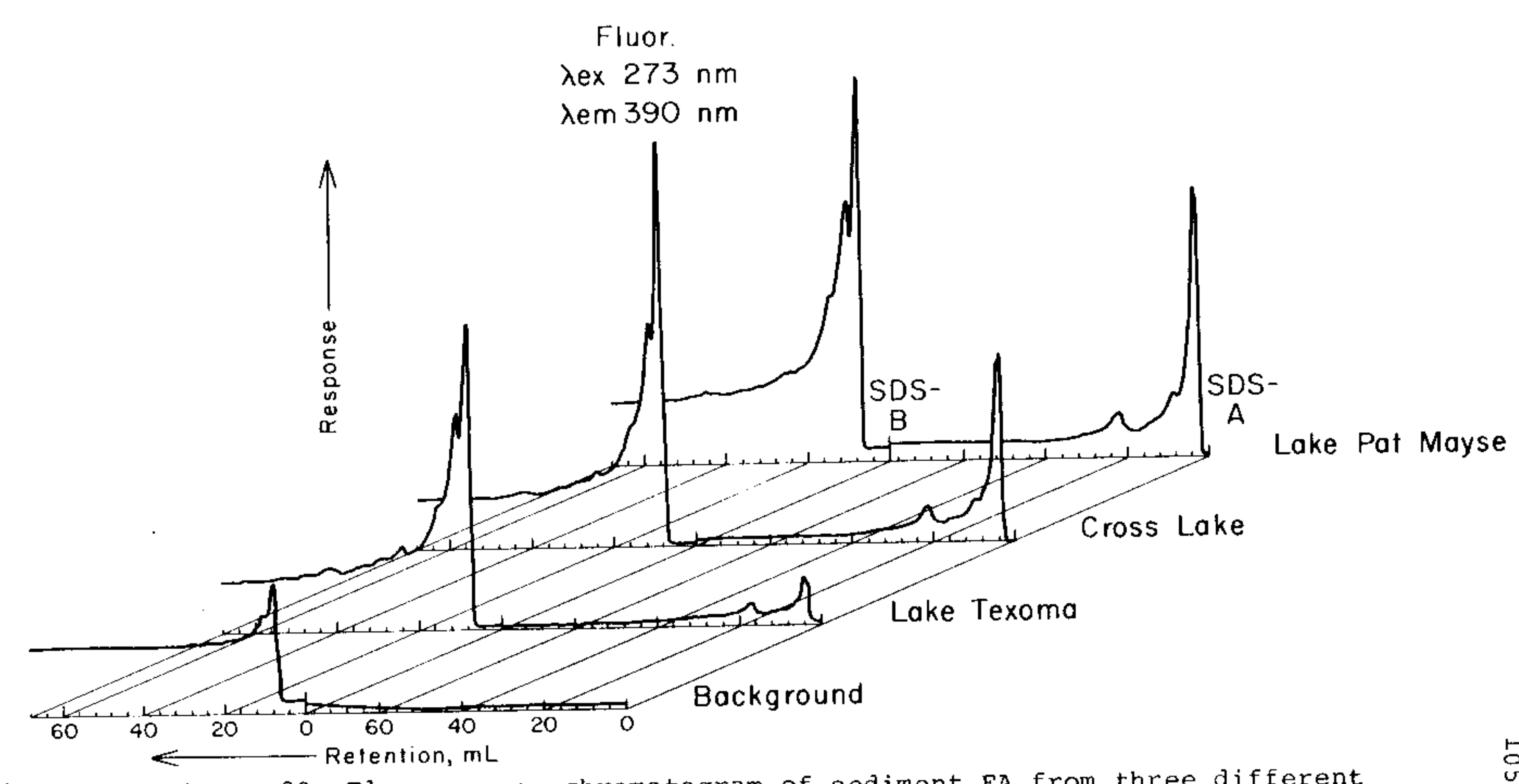

Figure 30--Fluorescence chromatogram of sediment FA from three different locations. Sample, Lake Pat Mayse, Cross Lake and Lake Texoma; eluent, SDS-A and SDS-B; the operating conditions same as described in Figure 16.

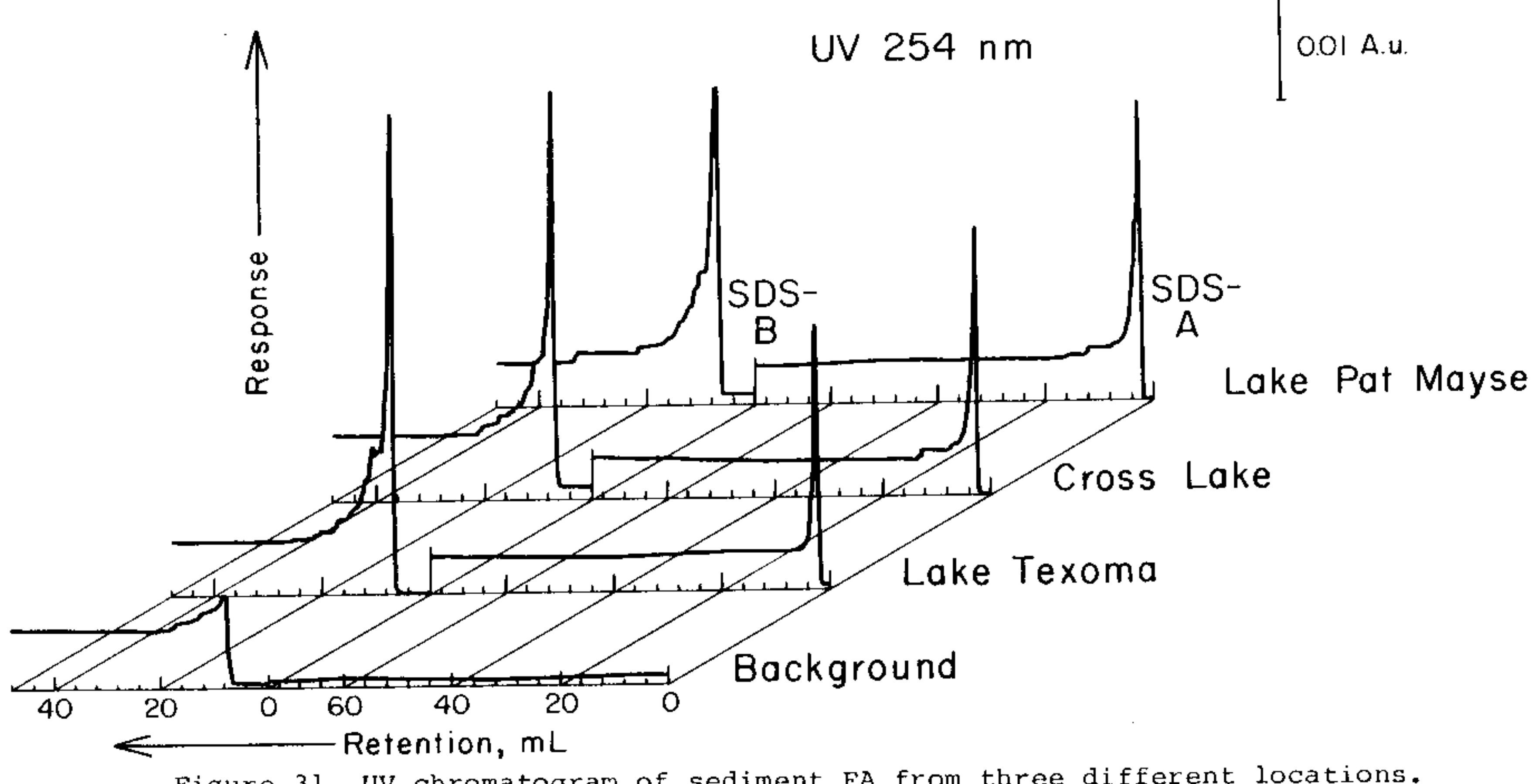

Figure 31--UV chromatogram of sediment FA from three different locations. Sample and the conditions same as described in Figure 30.

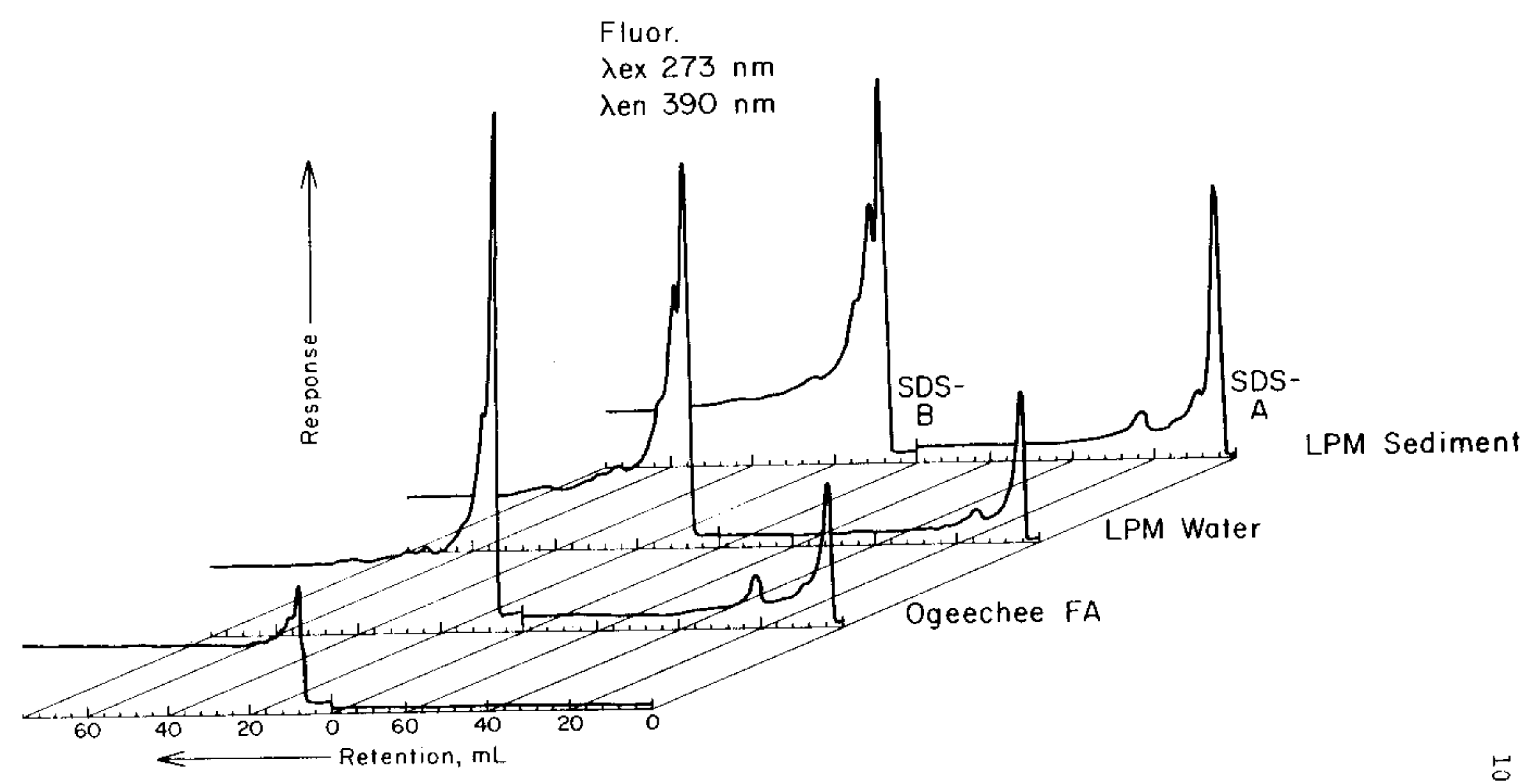

Figure 32--Fluorescence chromatogram of sediment, water and reference FA. Sample, Lake Pat Mayse sediment, water and Ogeechee reference FA; the conditions same as described in Figure 31.

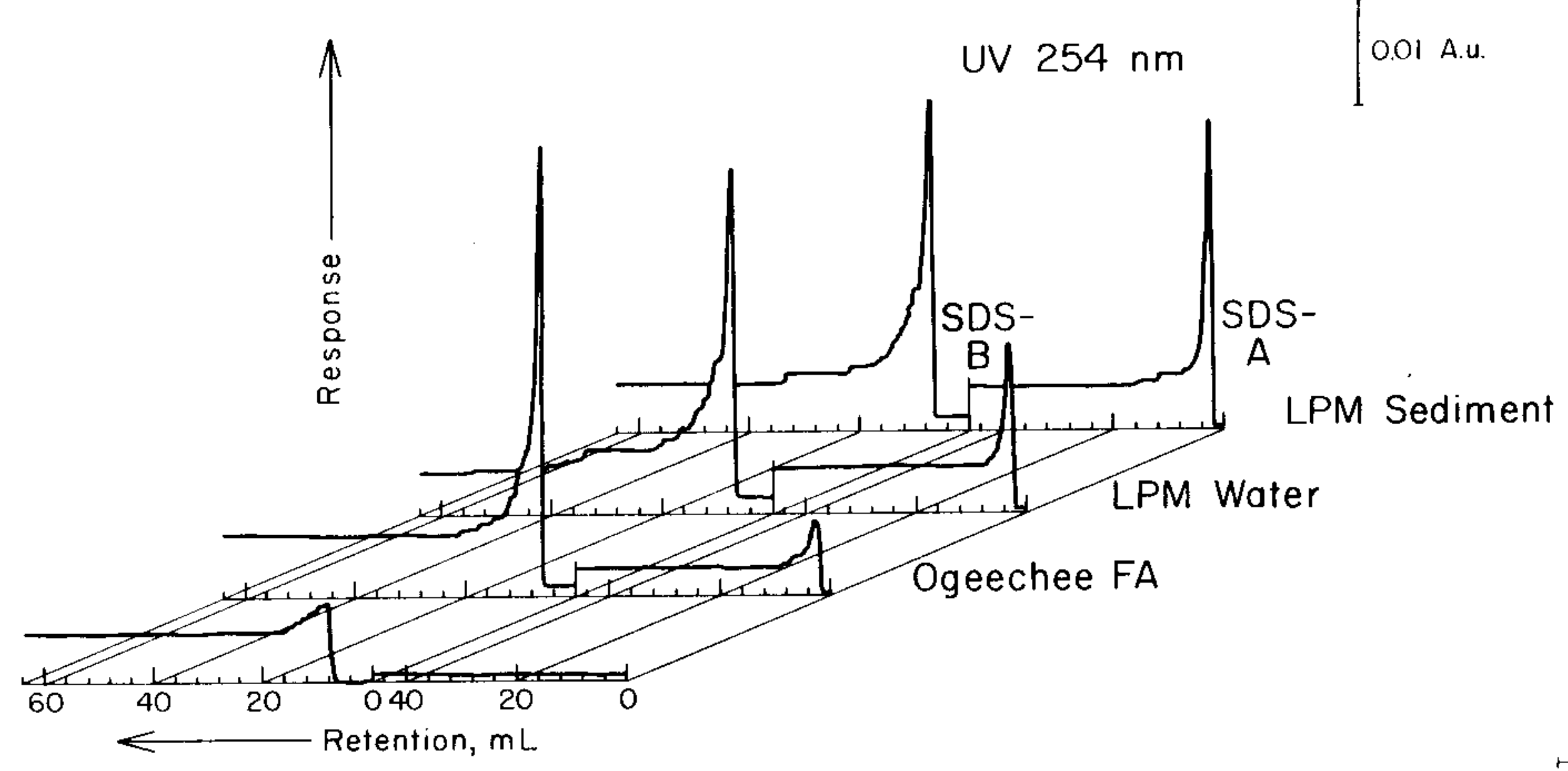

Figure 33--UV chromatogram of sediment, water and reference FA. Sample and the conditions same as described in Figure 32.

"ion-exchange" model adequately describes chromatographic phenomena observed in RP-HPLC systems containing surface-active ions intentionally added to the mobile phase. Rather, they have proposed an ion-interaction mechanism which does not require classic ion-exchange "sites" or ion-pair formation in either phase. Important features of the ion-interaction model are that (a) adsorbed surface active ions (ion-interaction reagent, IIR) are responsible for a charged primary ion layer at the surface of the stationary phase; (b) the charged primary ion layer electrostatically attracts or repels solute ions of opposite or similar charge, respectively; (c) the charged primary ion layer does not exert an effect upon uncharged molecules; (d) other differences in distribution behavior can be explained by forces that they are eluophilic, eluophobic, adsorbophilic, and adsorbophobic (39).

Figure 34 shows the u.v. and fluorescence chromatograms of Lake Pat Mayse sediment fulvic acid by adding ion-interaction reagent octylamine (pH 6.34) into the carrier solvent system. It is noted that four characteristic responses with k' 0.40, 0.72, 1.87, and 6.01 are resolved in the u.v. chromatogram. The increasing retention (k' 6.01) can be refered to the effect of ion-interaction reagent. At pH 6.34, a greater fraction of the fulvic acid exists in the unprotonated, negatively

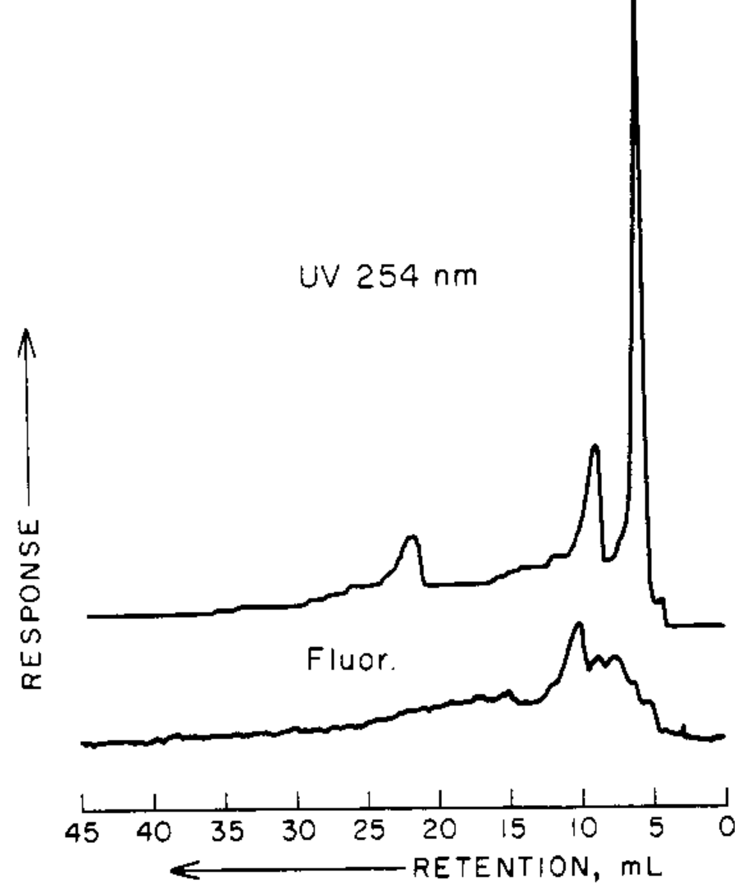

Figure 34--Chromatograms of sediment FA with ion-interaction reagent. Sample, Lake Pat Mayse sediment FA; eluent, octylamine solvent; column and the operating conditions same as described in Figure 15.

charged conjugate base form which strongly interacts with the positively charged adsorbed primary ion layer created by the adsorbed octylamine ion. This results in a increase in retention time.

Gas Chromatography

Highly specific degradation techniques which have been successful in elucidating the chemical structure of other natural products such as steroids, or proteins, were not successful with humic and fulvic acids (49). This may be attributed to the heterogeneity and polymeric nature of fulvic acids. Humic substance have proved to be highly resistant to chemical attack by many of the reagents that are used for partial degradation. Wershaw et al. (31) suggested that this resistance to chemical attack arises from the fact that humic substance form molecular aggregates in solution that must be disaggregated in order that specific functional groups in the humic substance molecules can be exposed to chemical reaction. Fulvic and humic acid molecules are formed from both homogeneous and heterogeneous aggregates. In order to determine the chemical structure of fulvic and humic acid, it is necessary to disrupt this aggregation and to isolate the various chemical species (49). These aggregates are held together by various weak-bonding mechanisms, the most important of which is hydrogen bonding between the carboxylic acid groups,

phenolic groups, and other hydroxylic groups. The reduction of this hydrogen bonding can be accomplished by replacing the protons in acidic, phenolic and alcoholic groups with methyl groups.

A two-step methylation procedure (49, 50) for fulvic acid was used in this research. In the first step, carboxylic acid groups were methylated with diazomethane in dimethylformamide (DMF); in the second step, hydroxyl groups were methylated with methyl iodide and sodium hydride in DMF. Figure 35 shows GC chromatograms of Suwannee reference fulvic acid methylated with diazomethane and without methylation by SE-30 capillary column and FID detection. Several peaks are found in the methylated sample. These can be attributed to the carboxylic acid and highly acidic phenolic moiety of fulvic acid which is methylated into methyl ester and resolved by capillary column. Figure 36 shows the GC chromatograms of Suwannee and Ogeechee reference fulvic acid methylated with methyl iodide and sodium hydride. The peaks found in this Figure can be related to the weakly acidic phenolic groups and carbohydrate alcoholic groups which do not react with diazomethane (49). Figure 37 shows the GC chromatograms of Suwannee reference fulvic acid with two-step methylation and derivatized procedure blank. The methylation method enables us to distinguish various OH groups in complex

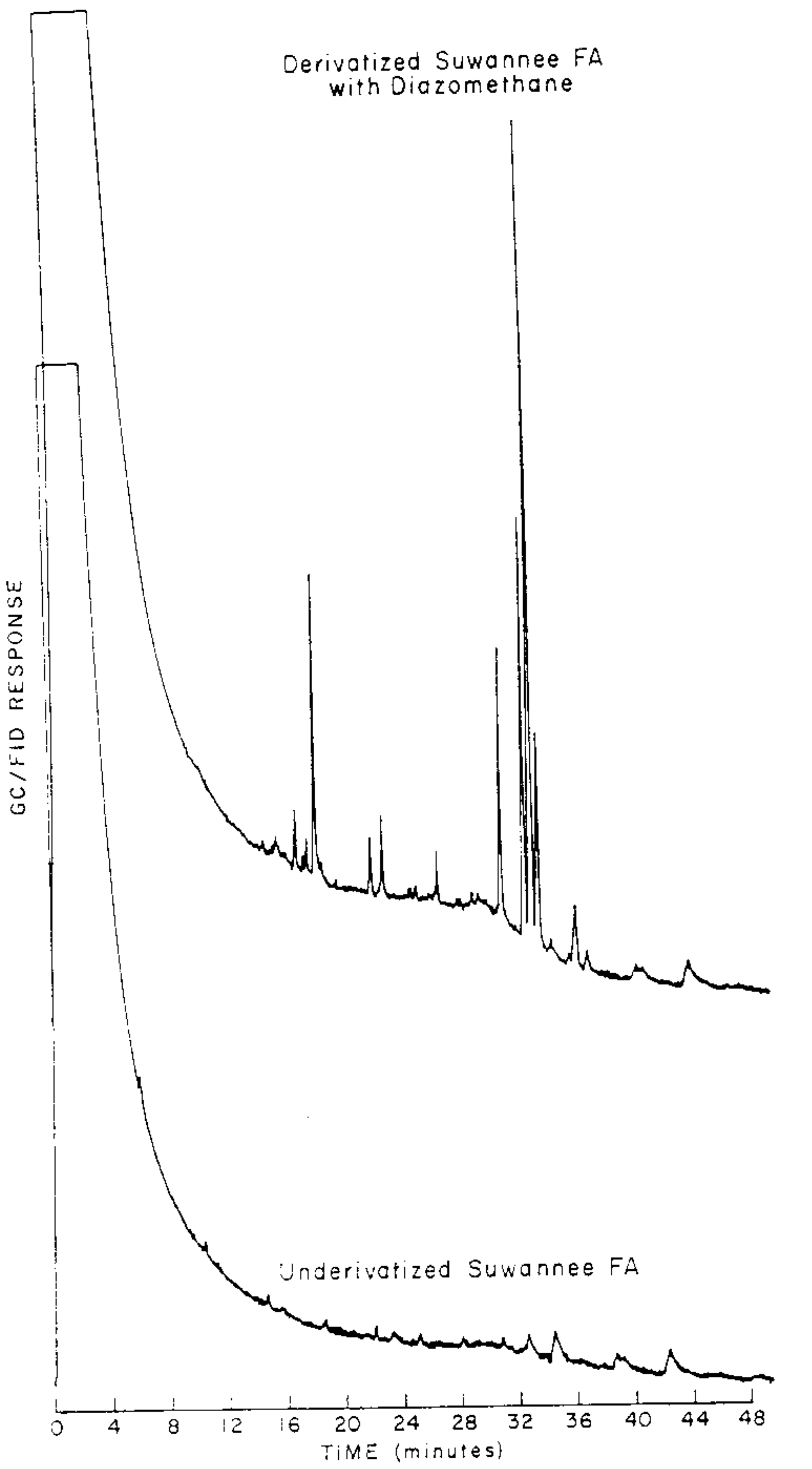

Figure 35--GC/FID chromatograms of reference FA with and without methylation. Sample, Suwannee FA; column, SE-30 capillary column; solvent, DMF; temperature 50 to 200 °C at 5 °C/min.

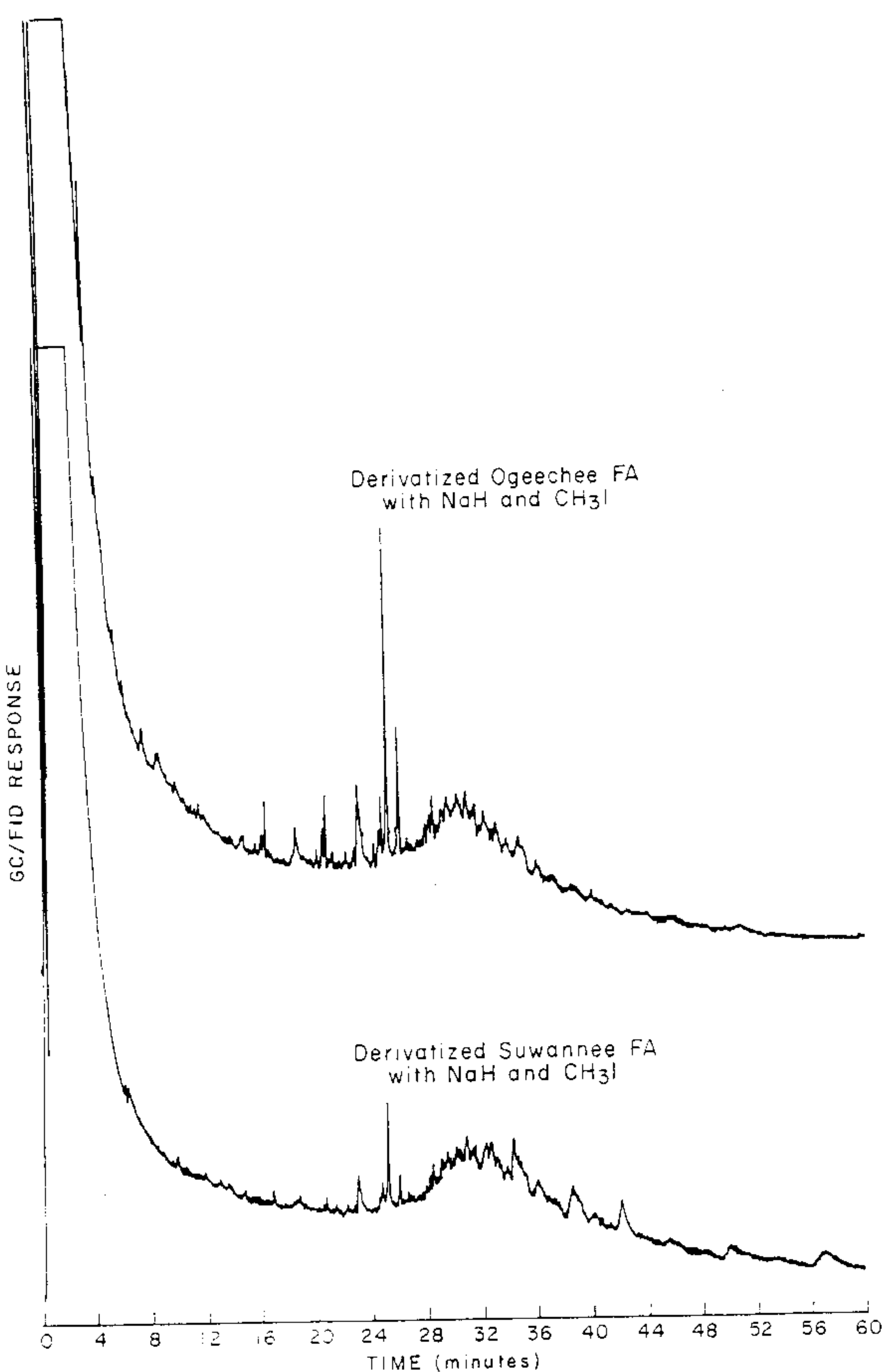

Figure 36--GC/FID chromatograms of two reference FA methylated with methyl iodide. Sample, Suwannee and Ogeechee reference FA; conditions same as described in Figure 35.

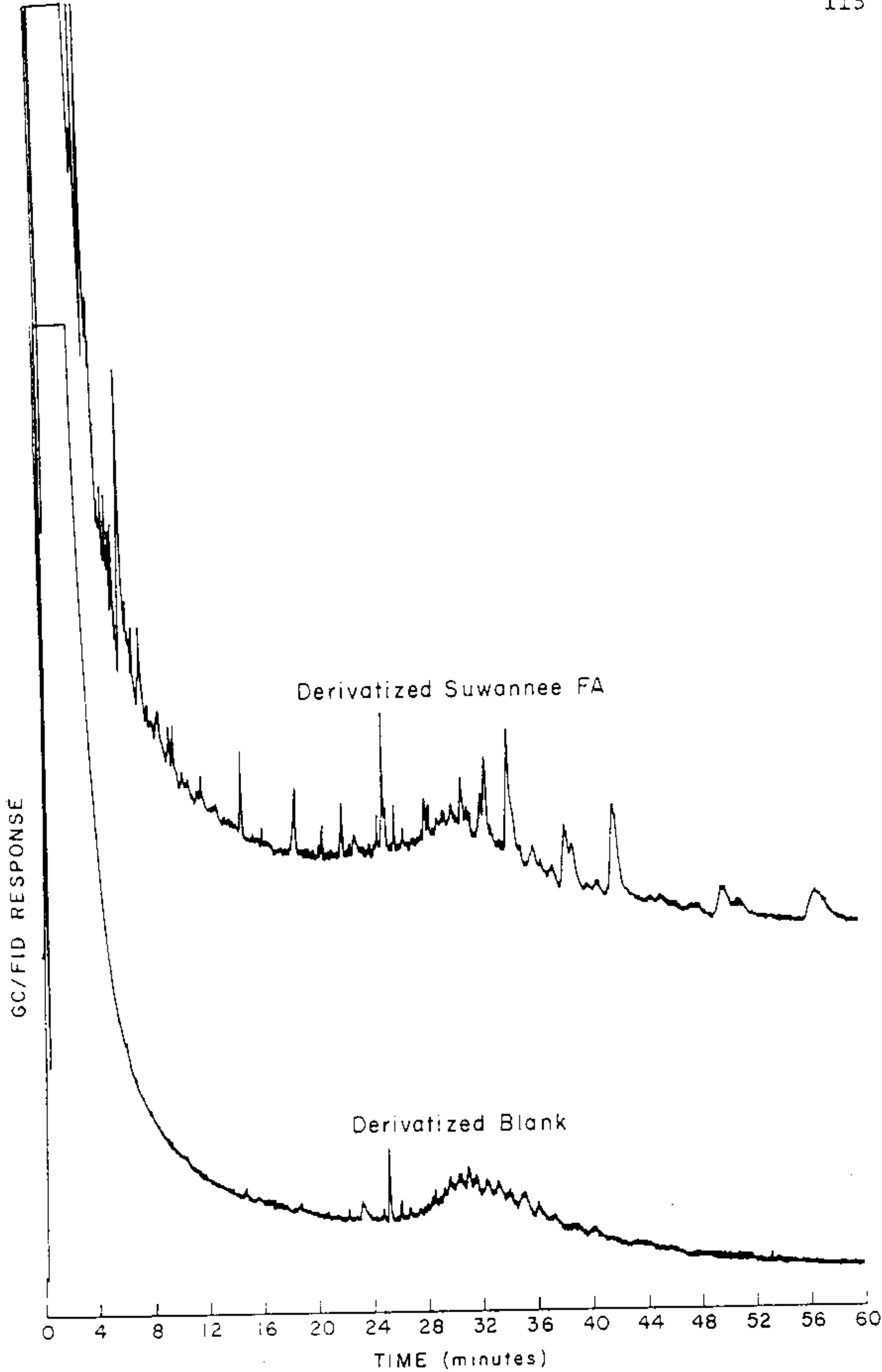

Figure 37--GC/FID chromatograms of FA with two-step methylation and procedure blank. Sample, Suwannee FA and procedure blank; conditions same as described in Figure 35.

macromolecule. No further efforts were made to identify the peaks as it was not a primary objective of the study.

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CHAPTER IV

CONCLUSION

The first objective of this research was to develop characteristic profiles of aquatic fulvic acid and to identify similarities and differences between samples collected from different locations. For this objective, three modes of HPLC: adsorption, anion exchange and reversed-phase were evaluated. Major efforts were focused on reversed-phase (RP) HPLC to investigate the similarities and differences between samples collected from different locations. Seven types of carrier solvent systems with wide range of polarity in the presence or absence ion pairing or organic modifiers, were used to optimize the separation conditions in RP-HPLC. Results have shown that aquatic fulvic acid extracted from sediment and water from different locations have common characteristic responses under different modes of HPLC with differences only in the magnitude of the u.v. and fluorescence responses.

Results from the anion exchange HPLC have shown that at least three types of acidic functionalities in aquatic fulvic acid can be separated. Results also indicate that aquatic fulvic acid can be progressively fractionated using subsequent modes of HPLC.

The second objective of this research was to compare the chromatographic characteristics of reference fulvic acid with those collected from different locations. Results of the reversed-phase (1, 2), part of this study have shown that aquatic fulvic acid from different locations are fractionated into the same major components under different RP-HPLC conditions. The polar components represented 50-70% of total u.v. and fluorescence response. The non or moderately polar components represented 30-50% of the total response and were resolved into 3-5 peaks. Results indicated that: (i) The fractionation of aquatic fulvic acid by RP-HPLC is essentially controlled by the polarity and/or pH of the carrier solvent system. (ii) Under different RP-HPLC conditions aquatic fulvic acids from different locations were fractionated into the same major components as determined by u.v. and fluorescence response. Differences between the major components involved the intensity of the u.v. absorption or fluorescence response and the fine resolution of each major component. (iii) Fulvic acid extracted from water and sediment from the same site are more similar than those extracted from different sites.

Under isocratic and stepwise conditions, using cationic and anionic ion pair reagents, fulvic acid were eluted and showed resolutions of 8-9 peaks indicated the presence of amphoteric compounds within the polymeric structure of

fulvic acid. The development of such characteristic profiles allows the identification of elements of homogeneity in the fulvic acid macromolecules. The importances of these results relate to future investigation of the structure of fulvic acid.

The third objective of this research is to fractionate components of reference fulvic acid on a semipreparative scale using a suitable HPLC mode for further investigation by other spectroscopic methods. For this objective, Solv. A1 and B1 was selected from the seven types of carrier solvent systems used. This solvent system indicated well defined separation of two major components of fulvic acid. The total amount of 70 mg Suwannee reference fulvic acid was fractionated into two major fractions by using ST/C_{18} semipreparative scale column under stepwise gradient condition. Fractions are kept for further spectroscopic measurement. Furthermore, a major fraction of Cross Lake fulvic acid collected by adsorption chromatography was subjected to CP-MAS Carbon-13 NMR and FAB Mass Spectroscopy.

Results (3) indicated that (i) The analyzed fraction of fulvic acid contains more aliphatic than aromatic moieties. The aliphatic moieties are represented by branched aliphatics with methylene carbons α, β, or γ from a terminal methyl group or an aromatic ring. The aromatic moieties include several substituted aromatics with structures that contain metal complexing sites and are potential precursors

of THMs upon chlorination. (ii) Methoxy, carboxylic acids, and esters are well-defined moieties of the macromolecule. Their presence is indicated by both Carbon-13 NMR and FAB-MS. (iii) Phenolic components of the macromolecules were not detected in the Carbon-13 NMR spectrum possibly because of the presence of stable free radicals. Several phenolic structures were detected in the FAB-MS.

The gas chromatographic methylation method enables to distinguish various OH groups in complex macromolecules. The carboxylic acid and highly acidic phenolic moiety of fulvic acid can be methylated into ester with diazomethane. The weak acidic phenolic group and carbohydrate alcoholic group can be methylated with methyl iodide.

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