Concentration and Fractionation of Hydrophobic Organic Acid Constituents from Natural Waters by Liquid Chromatography

GEOLOGICAL SURVEY WATER-SUPPLY PAPER 1817–G

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ORGANIC SUBSTANCES IN WATER

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By E. M. THURMAN and R. L. MALCOLM

ABSTRACT

A scheme is presented which used adsorption chromatography with pH gradient elution and size-exclusion chromatography to concentrate and separate hydrophobic organic acids from water. A review of chromatographic processes involved in the flow scheme is also presented. Organic analytes which appear in each aqueous fraction are quantified by dissolved organic carbon analysis. Hydrophobic organic acids in a water sample are concentrated on a porous acrylic resin. These acids usually constitute approximately 30–50 percent of the dissolved organic carbon in an unpolluted water sample and are eluted with an aqueous eluent (dilute base). The concentrate is then passed through a column of polyacryloylmorpholine gel, which separates the acids into high- and low-molecular-weight fractions. The high- and low-molecular-weight eluates are reconcentrated by adsorption chromatography, then are eluted with a pH gradient into strong acids (predominately carboxylic acids) and weak acids (predominately phenolic compounds). For standard compounds and samples of unpolluted waters, the scheme fractionates humic substances into strong and weak acid fractions that are separated from the low molecular weight acids. A new method utilizing conductivity is also presented to estimate the acidic components in the methanol fraction.

INTRODUCTION

To effectively study most natural and anthropogenic organic substances in water, they must be concentrated and separated. Because the concentration of organic substances in water by DOC (dissolved organic carbon) analysis is from 1–20-mg/L (Malcolm and Durum, 1976), trace enrichment is necessary. Liquid chromatography is an analytical technique that can be very efficient in both the concentration and isolation of natural and anthropogenic organic analytes. This paper deals with the utilization of two types of liquid chromatography: liquid-solid adsorption chromatography with a porous acrylic resin, and size exclusion chromatography with a polyacryloylmorpholine gel, to concentrate and classify hydrophobic organic acid con-

stituents from water. The use of these two forms of liquid chromatography coupled with carbon analysis for detection allows a meaningful grouping of hydrophobic organic acid solutes into seven fractions. The mass of carbon in each fraction is quantified by DOC analysis. Although specific compound identification has not yet been accomplished, it is greatly facilitated by preconcentration and may be done on fractions of interest.

The flow scheme combines various chromatographic procedures which includes the adsorption process (Malcolm and others, 1977; Thurman and others, 1977; Thurman and others, 1978; Aiken and others, 1977), size exclusion, and the most recent work with gradient elution and HPLC (high-performance liquid chromatography) with macroporous resins. The purpose of this report is to discuss integration of these various techniques and to develop a flow scheme for evaluation of hydrophobic-organic-acid constituents in a natural water sample.

ACKNOWLEDGMENTS

Appreciation is expressed to United States Geological Survey personnel who made this study possible.

EXPERIMENTAL PROCEDURES

RESIN AND COLUMN PACKINGS

Amberlite[1] XAD–8, a porous acrylic ester, was obtained from Rohm and Haas Chemical Co. The 40- to 60-mesh resin was extensively cleaned with 0.1 *N* NaOH and sequentially extracted with methanol, ether, acetonitrile, and methanol for three days each, then stored in methanol. Medium grade KO Enzacryl[1] gel that has a polyacryloylmorpholine structure was obtained from Aldrich Chemical Co. The cleaning procedure was similar to that for XAD–8, except only NaOH and methanol were used. The gel was stored in methanol to prevent bacterial growth.

INSTRUMENTATION

A Varian Aerograph 2700 gas chromatograph, Varian 8500 liquid chromatograph, Beckman 915 carbon analyzer with 215A infrared detector, and a Dohrmann 52D carbon analyzer with flame-ionization detector were used for sample and standard organic analysis. Peristaltic pumps were used for low-pressure systems with Glenco glass

[1]The use of the brand names in this report is for identification purposes only and does not imply endorsement by the U.S. Geological Survey.

columns, 3500 series. A Perkin Elmer Model 305B atomic absorption spectrophotometer was used for sodium analysis.

REAGENTS

All reagents were analyzed reagent grade. The sodium hydroxide, hydrochloric acid, and phosphoric acid were obtained from J. T. Baker Chemical Co. The tannic acid was obtained from Mallinckrodt Chemical Co. The polyacrylic, hexanoic, and heptanoic acids were purchased from Aldrich Chemical Co. Benzoic acid, phenol, nonanoic acid, p-chlorophenol, l-naphthol, and p-nitrophenol were obtained from Chem Services. Finally, the Seward fulvic acid and North Carolina humic acid were extracted from soils near Seward, Alaska, and Wilmington, N. C., by Ronald Malcolm, U.S. Geological Survey, Denver, Colo.

COLUMN PROCEDURES

ADSORPTION CHROMATOGRAPHY

The XAD–8 resin was packed as a methanol and water slurry into a 93-mL column, then rinsed with deionized water until free of methanol. This required approximately 50 bed volumes of water before a blank DOC analysis was obtained. A final rinsing of the packed column with two cycles of 0.1 *N* NaOH and 0.1 *N* HCl removed traces of monomers (acrylic acid) and soluble-uncrosslinked polymers from the resin. A 10-liter sample of the South Platte River was filtered through a 0.45-micrometer silver filter, acidified to a pH of two with HCl, and pumped through the column at a flow rate of 10 bed volumes per hour (15 mL/min). The column effluent was collected in a glass bottle and refrigerated for further study. The column was then eluted with three bed volumes (300 mL) of 0.1 *N* NaOH, the effluent was monitored at a wavelength of 254 nanometers, and 10-ML fractions were collected. Next the eluent was changed to deionized water and three bed volumes were collected; finally, three bed volumes of methanol were passed through the column and collected. This resulted in approximately complete recovery of the adsorbed acid constituents, as checked by DOC analysis.

SIZE EXCLUSION CHROMATOGRAPHY

Enzacryl gel was packed as a methanol and water slurry into a 288-mL column and was rinsed with deionized water until free of methanol (20 bed volumes); then the mobile phase was adjusted to 0.1 *N* NaOH. Approximately three bed volumes of NaOH were run through the column before the effluent had a blank carbon level, which was less than 5-mg/L DOC. A 2-mL sample of the NaOH eluate

from the adsorption column was pipetted onto the head of the Enzacryl column and carefully loaded into the column using the NaOH mobile phase. The column was then pumped for one hour at a rate of one bed volume per hour (5-mL/minute). The effluent was monitored at 254 nm and was collected in 10-mL fractions.

pH GRADIENT CHROMATOGRAPHY

A 4.2-mL column of XAD–8 was used for the pH gradient chromatography. The eluate from the gel filtration column was concentrated by adjusting the pH to two with phosphoric acid, and pumping the eluate onto the XAD–8 column at a rate of 10 bed volumes per hour (0.7 mL/min). The pH gradient consisted of two solutions at a pH of 7.2 and 13. The lower pH ionized only the carboxylic acids present in the sample and eluted those first. The higher pH eluted the weaker acids such as phenols. A 0.1 *M* phosphate buffer was used for the pH 7.2 mobile phase, and 0.1 *N* sodium hydroxide was used for the pH 13 solution. Three bed volumes of each buffer were passed through the pH gradient column.

FRACTIONATION OF STANDARD COMPOUNDS

Thirty-five test compounds were examined one at a time and in groups of two or three compounds. The concentration of test compounds was 1- to 10-mg/L DOC. Recoveries were 90–100 percent by DOC analysis and gas chromatography.

REVIEW OF CHROMATOGRAPHIC PRINCIFLES OF FLOW SCHEME

GENERAL FLOW SCHEME

The fractionation scheme which is presented consists of three main steps (fig. 1). First, there is an adsorption procedure to enrich trace amounts of organic acids present in water samples. The adsorption procedure concentrates both organic acids and neutrals of hydrophobic character. The term hydrophobic as used in this paper is based on the solubility of an organic solute in water. A simple organic solute which is only slightly soluble, 0.01 moles per liter maximum solubility, will be hydrophobic, or concentrated on the resin (Thurman and others, 1978). Organic acids are those solutes which contain either carboxylic or phenolic hydroxyl functional groups. Neutral solutes are those which either do not contain functional groups or contain functional groups which are not ionic in weak base. Elution of this column with weak base then methanol fractionates hydrophobic compounds into hydrophobic acids and neutrals, respectively.

The second step in the fractionation scheme is size-exclusion

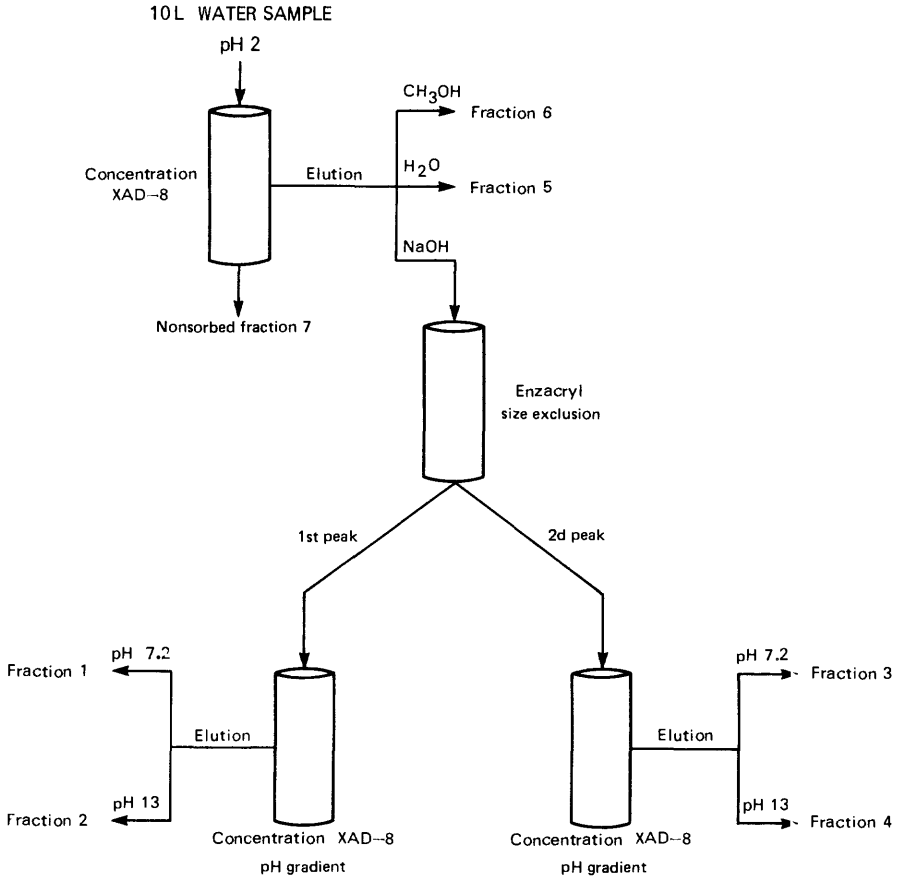

FIGURE 1.—Flow scheme.

chromatography. This procedure is used to separate the hydrophobic acid fraction into low- and high-molecular-weight groups. Hydrophobic acids are fractionated by size in their ionic state with 0.1 *N* NaOH as the mobile phase; this greatly reduces interaction between gel and solute, in order that molecular exclusion is the major separation factor.

Finally, in step three, adsorption chromatography is used to concentrate hydrophobic acids which are diluted in the gel permeation step. Gradient elution is used to separate strong and weak acids. The strong acids are eluted with a phosphate buffer at pH 7.2, and the weak acids in 0.1 *N* NaOH at pH 13.

Through the entire procedure, mass balance of carbon is monitored with DOC analysis. Because the solvents used do not contain carbon, (with the exception of methonol), this is easily accomplished. A detailed explanation of each step follows.

ADSORPTION CHROMATOGRAPHY

ADSORPTION PROCESS

It is known that physical adsorption, such as Van der Waals' forces, is a principal factor in the adsorption process of aqueous organic solutes onto nonionic resins, with enthalpies of adsorption from 2 to 6 (kcal/mole) kilocalories per mole (Gustafson and Paleos, 1971). Hydrogen bonding and dipole-dipole interactions are thought to occur, especially in nonaqueous solvents (Simpson, 1972). Solutes must be nonionic to sorb efficiently because of the strong ion-dipole bond which will form between the ionized solute and the water molecule. Grieser and Pietrzyk (1973) present a mathematical relationship which shows that the distribution coefficient of any compound for XAD–2 depends on the fraction of that compound in the nonionic state.

Isotherm data are reported for many organic acids on XAD-resins (Gustafson and Paleos, 1971; Simpson, 1972; Gustafson and others, 1968). Gustafson and Paleos (1971) report that Langmuir isotherms are nonlinear over a wide range of concentration but are linear at low concentrations, less than 10^{-3} (mol/L) moles per liter. They also found that Freundlich isotherms fit adsorption curves of many organic acids by XAD-resins. The above isotherm data suggest that at low concentrations of organic acids in water ($10^{-=}$ fififi/L), adsorption isotherms are essentially linear for XAD-resins.

Thurman, Malcolm, and Aiken (1978) have shown that organic acids containing five carbons per carboxyl functional group are hydrophobic enough to be retained by XAD-resins when concentrated from dilute natural waters. For example, simple fatty acids from formic to butyric are hydrophilic and pass through XAD-resins even in the nonionic state. A relationship between column- and water-sample size is used to predict which organic acids are retained. Finally, solubility is used to estimate the capacity factor of an organic solute for XAD–8 resin.

Generally, DOC of natural surface waters varies from 5- to 20-mg/L DOC. Approximately 40–60 percent of this DOC can be removed by adsorption onto the resins when a capacity factor, k', of 250 is used to adjust column and sample size (Malcolm and others, 1977). Organic solutes that are not retained are called hydrophilic, and those that are retained, hydrophobic. In natural samples it is found that the hydrophilic-hydrophobic break is gradational.

ELUTION PROCESS

Elution of concentrated organic acids begins with 0.1 *N* NaOH as the eluent (fig. 1). This fraction is considered the main fraction of the

flow scheme. Organic solutes containing carboxylic and phenolic hydroxyl groups are ionic in 0.1 *N* NaOH at a pH of 13. The capacity factor of organic acids decreases to approximately one in dilute base, and the solutes elute. Typically, 50–80 percent of the hydrophobic solutes elute in this fraction (Malcolm and others, 1977; Thurman and others, 1977), and most of the humic substances are removed by the 0.1 *N* NaOH (Aiken and others, 1977). However, there is a point where interaction of the ionic functional group with the solvent, water, is not energetically sufficient to desorb the hydrophobic portion of the molecule from the resin, and the hydrophobic ion remains adsorbed. From our preliminary data and other oral communication with H. A. Stuber (U.S. Geological Survey, Denver, Colo., 1978), when the ratio of carbon atoms to ionic functional groups is from 8:1 to 10:1, elution in 0.1 *N* NaOH is poor. Thus, a simple fatty acid such as nonanoic acid begins to show retention on XAD–8 resin in 0.1 *N* NaOH, and decanoic acid will not elute in a reasonable volume of 0.1 *N* NaOH (three bed volumes).

The effect of ion-pair adsorption on the elution process is presently under intensive investigation with the following tentative conclusions. It has been noted that ionic strength influences retention of the organic ion (oral commun., H. A. Stuber, 1978) on XAD-resins. As ionic strength of the solution goes up, the organic solute is "salted out" or retained. Therefore, in this flow scheme, water is used as an eluent after 0.1 *N* NaOH . The large drop in ionic strength from 0.1 to 0.001 elutes a second peak, which represents compounds that contain a very narrow ratio of carbon atoms to ionic functional groups (approximately 8–10 carbon atoms per functional group). Organic acids with more than 10 carbons per functional group are retained on the column, whereas compounds with less than 8 carbons per functional group are removed in the 0.1 *N* NaOH.

ENZACRYL GEL CHROMATOGRAPHY

Enzacryl gel was chosen for size exclusion chromatography because it is chemically inert, it is stable at high pH, and it is not susceptible to attack by microorganisms (Epton and Holloway, 1978). The major mechanism of separation is thought to be size exclusion. Larger organic acids cannot penetrate the gel, pass quickly through the column, and emerge as the first elution peak. Smaller organic acids migrate into the gel matrix, are retained, and are eluted as the second peak. The mobile phase, 0.1 *N* NaOH, is used to ionize the acids and to minimize solute-gel interactions. However, there is also the following evidence that charge exclusion and adsorption mechanisms are involved. Small fatty acids which are charged, such as acetic acid, oxalic acid, and citric acid, do not penetrate the gel completely and

are only partially separated from high-molecular-weight humic substances. Chloro-substituted and nitro-substituted phenols are retained slightly longer than the bed volume of the gel, indicating slight adsorption. All the nonhumic-hydrophobic acids tested on the gel (approximately nine compounds), were separated from the humic substances, regardless of the proposed mechanism (table 1, fractions 3 and 4).

ADSORPTION CHROMATOGRAPHY WITH pH GRADIENT ELUTION

The two fractions from the size-exclusion step are acidified and concentrated on two XAD–8 columns. Elution begins at a pH of 7.2, which causes elution of carboxylic acids, because the pK_a (negative logarithm of the acid-dissociation constant) of these acids is between four and five. Next, the gradient is stepped to 0.1 *N* NaOH at pH 13, and the phenolic hydroxyls (weaker acids) are ionized and eluted. More elaborate pH gradients have been attempted, such as titration of a 0.1 *M* phosphoric acid with 0.1 *N* NaOH. A gradient is produced with three buffer zones. However, the separation of natural acids is not facilitated by this procedure, and the same two major groups are found.

This concludes the flow scheme. Seven fractions are obtained; these include a high- and low-molecular-weight fraction, which are carboxylic acids; a high- and low-molecular-weight fraction, which are predominately weaker acids; a water fraction; a methanol fraction; and a nonsorbed fraction.

RESULTS AND DISCUSSION

STANDARD COMPOUND FRACTIONATION

Table 1 shows the fractionation of 35 test compounds, according to the flow scheme shown in figure 1. Humic, fulvic, and tannic acids are the natural high-molecular-weight acids (500–2,000 molecular weight), which were separated into fractions 1 and 2. Fraction 1 contains standards which are stronger carboxylic acids; whereas, fraction 2 is weakly acidic and contains phenolic hydroxyl groups (tannic acid). Fraction 3 contains test compounds of low-molecular-weight (150) and carboxylic-acid functional groups. Fraction 4 consists of weak, low-molecular-weight acids. Fraction 5 is eluted with water, and represents a narrow range of compounds with approximately 8–10 carbon atoms per functional group. Fraction 6 is the methanol fraction that contains long-chain fatty acids and hydrophobic neutrals. Finally, fraction 7 is the nonsorbed, hydrophilic material, which consists of test compounds of high solubility (very soluble to

TABLE 1.—*Standard compound fractionation*

Fraction 1[1]	Fraction 2	Fraction 3	Fraction 4	Fraction 5	Fraction 6	Fraction 7
Seward fulvic acid	Tannic acid	Benzoic acid	Phenol	Nonanoic acid	Palmitic acid	Acetic acid
North Carolina humic acid (65 percent)	North Carolina humic acid (35 percent)	Valeric acid	p-chlorophenol	Decanoic acid	Stearic acid	Proprionic acid
		Caproic acid			p-methylbenzyl-alcohol	Formic acid
Polyacrylic acid		Heptanoic acid			Benzene	Butyric acid
North Carolina fulvic acid		Octanoic acid			Toluene	Oxalic acid
		Cyclohexane Carboxylic acid			Benzaldehyde	Phthalic acid
		p-toluic acid			Benzyl alcohol	Ethanol
						Glucose
						Sucrose
						Butanol
						Pyridine

[1]Fraction number refers to figure 1.

miscible in water) with acidic, basic, or neutral functional groups. On the basis of the fractionation of these test compounds and previously discussed resin studies, the following interpretations are made on a natural sample.

FRACTIONATION OF A SOUTH PLATTE RIVER SAMPLE

ADSORPTION CHROMATOGRAPHY

After preconcentration of the sample, elution commenced with 0.1 *N* NaOH followed by water. The 0.1 *N* NaOH and water elution resulted in recovery of 18.4 and 4.5 percent of the DOC, respectively, (table 2). Ninety percent of the DOC eluted by the 0.1 *N* NaOH appeared in the first bed volume. The base eluate contained one colored peak; water contained two colored components. The brown component in the 0.1 *N* NaOH is the major humic fraction. The absorbance of this peak at 460 nm was directly correlated to DOC concentration.

A second brown peak was eluted in the water fraction. This fraction is interpreted as humic substances, with fewer functional groups per carbon atom than the humic fraction eluted with base. The water fraction is thought to represent a "salted-out" fraction in the 0.1 *N* NaOH; this "salted-out" fraction has been noted in the past in gel

TABLE 2.—*DOC fractionation data for South Platte River sample*

Fraction	Organic carbon (mg)	Dissolved carbon (percent)
Adsorption step 1		
0.1*N* NaOH[1]	35	18.4
Water[1]	8.5	4.5
Methanol[1]	76.5	40.3
Size-exclusion step 2		
High-molecular-weight	29.8	15.7
Low-molecular-weight	5.2	2.7
Adsorption with pH gradient step 3		
High-molecular-weight fraction strong acid	25.3	13.3
High-molecular-weight weak acids	4.5	2.4
Low-molecular-weight strong acids	5.0	2.6
Low-molecular-weight weak acids	.2	.1
Hydrophilic components		
Material not adsorbed on column[1]	70	37.5
Total	190	100

[1]These fractions add up to the total amount of dissolved organic carbon.

filtration (Baham and others, 1978). The second colored peak in water elution was green; this fraction exhibited a peak at 630 nm, which is coincident with chlorophyll *c*. Chlorophyll *c* is found in many diatoms ubiquitous in natural surface waters; and chlorophyll *c* has a structure which is compatible with the water fraction, that is, one functional group per 12 carbon atoms. Lack of solubility of the green component in 0.1 *N* NaOH is a "salting-out" effect; standards of chlorophyll *c* are being prepared by diatom culture to confirm this interpretation.

The methanol fraction contained 77 mg organic carbon by difference. The difference method is the amount of organic carbon remaining on the column after subtracting the NaOH and water eluates from the amount of organic carbon originally adsorbed. Methanol was the last eluent used; two colored peaks were found. The first was a green material which had a similar spectrum to that which eluted in water; the second was a brown peak. Acids with greater than 10–12 carbon atoms per hydrophilic functional group are retained on the column after 0.1 *N* NaOH and water elution; sodium atoms are associated with these acids to maintain electrical neutrality. Therefore, upon elution of these acids with methanol, sodium counterion is also eluted, and it can be monitored by either atomic-absorption spectrometry or specific conductivity. Sodium and conductivity monitoring during methanol elution gave three peaks (fig. 2). The total amount of sodium found was 0.054 millimoles, which represents 0.054 millimoles of acidic functional groups. The assumption of a 20:1 carbon atom to functional group ratio results in 13 mg of carbon. Based on this assumption, 17 percent of the organic carbon in the methanol fraction are acidic components.

SIZE EXCLUSION CHROMATOGRAPHY

The 0.1 *N* NaOH eluate was fractionated on an Enzacryl column with 95–100 percent recovery by DOC analysis (fig. 3). Eighty-five percent of this eluate appears in the first fraction (high-molecular-weight) and 15 percent in the second fraction (low-molecular-weight). The monitoring of absorbance at 254 nm did not show the second peak. The majority of the organic carbon which elutes from the XAD–8 column in 0.1 *N* NaOH appears to be "humic substances" and of high-molecular-weight. The low-molecular-weight fraction has not been characterized; therefore, its importance in natural samples is not evaluated.

ADSORPTION CHROMATOGRAPHY WITH pH GRADIENT ELUTION

The two fractions from the size exclusion column were concentrated again on XAD–8 resin after acidification to a pH of 2 with phosphoric

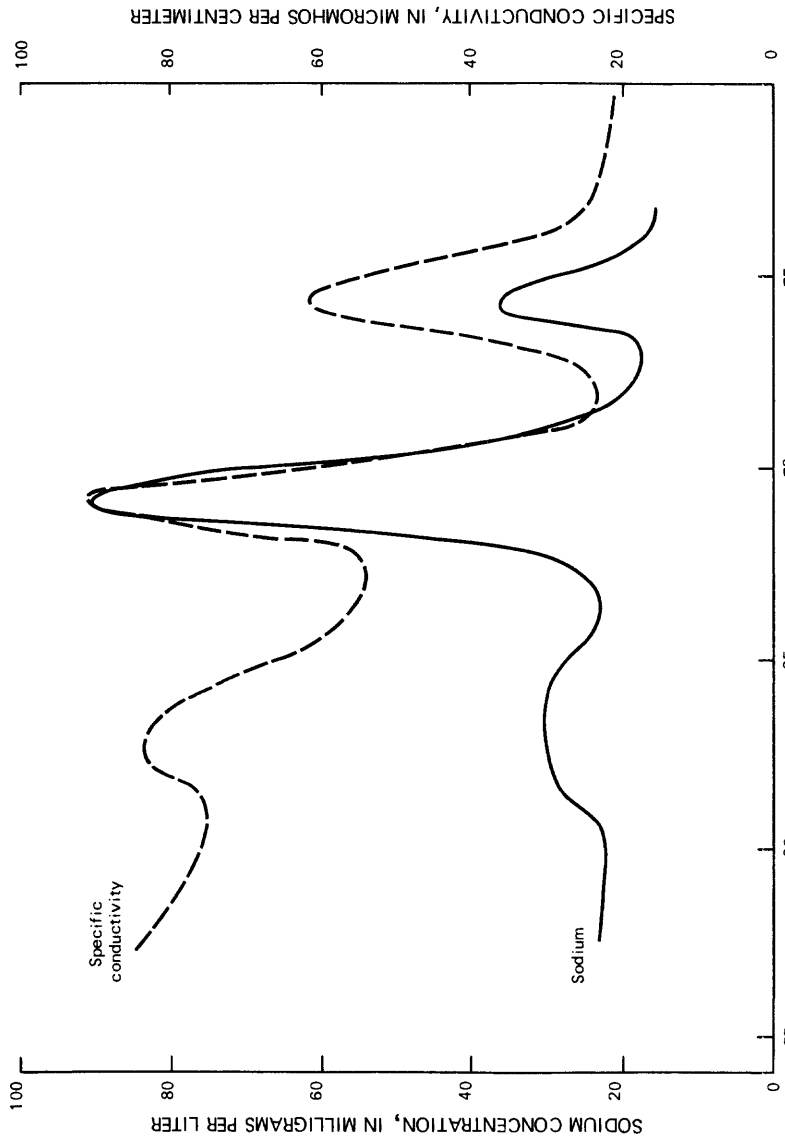

FIGURE 2.—Chromatogram of the methanol elution of the South Platte River sample.

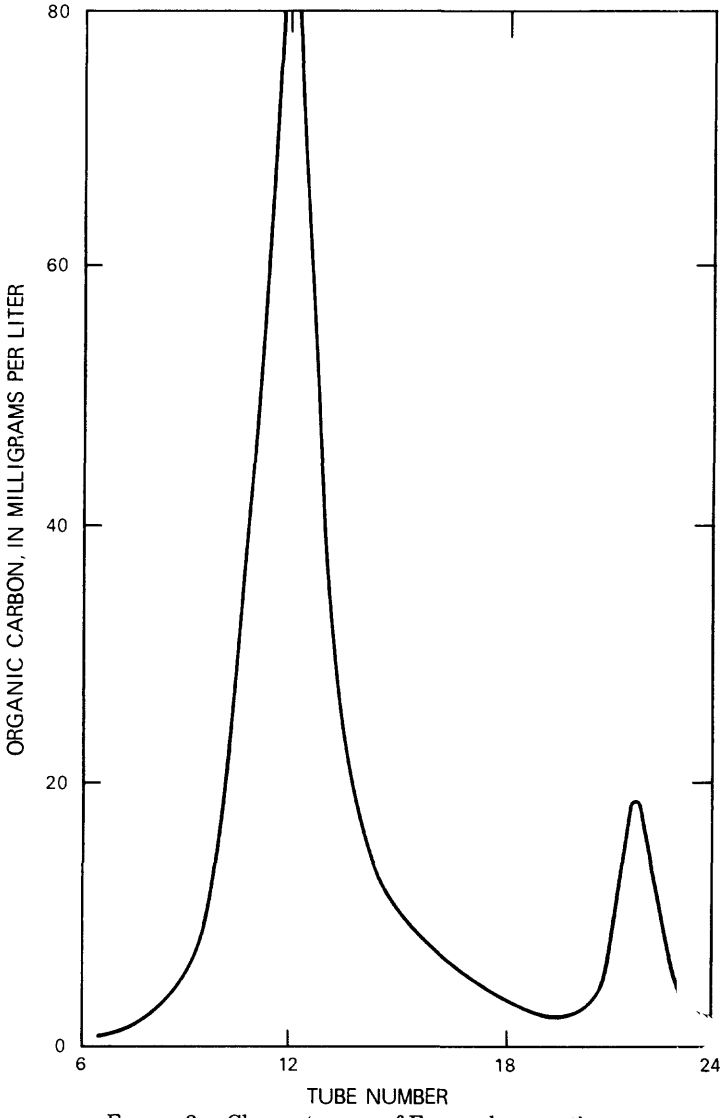

FIGURE 3.—Chromatogram of Enzacryl separation.

acid (fig. 1). Each was eluted with buffered solutions at a pH of 7.2 and 13. Both the high-and-low-molecular-weight fractions gave two peaks (fig. 4). In the high-molecular-weight fraction, 85 percent of the DOC were carboxylic acids (eluted at pH 7.2) and 15 percent were weaker acids (eluted at pH 13). The strong acid material was surfactant-like; the weak acids were not. The high-molecular-weight carboxylic acid fraction is postulated to be predominately fulvic acid. The weaker acid fraction is thought to be polyphenolic in character,

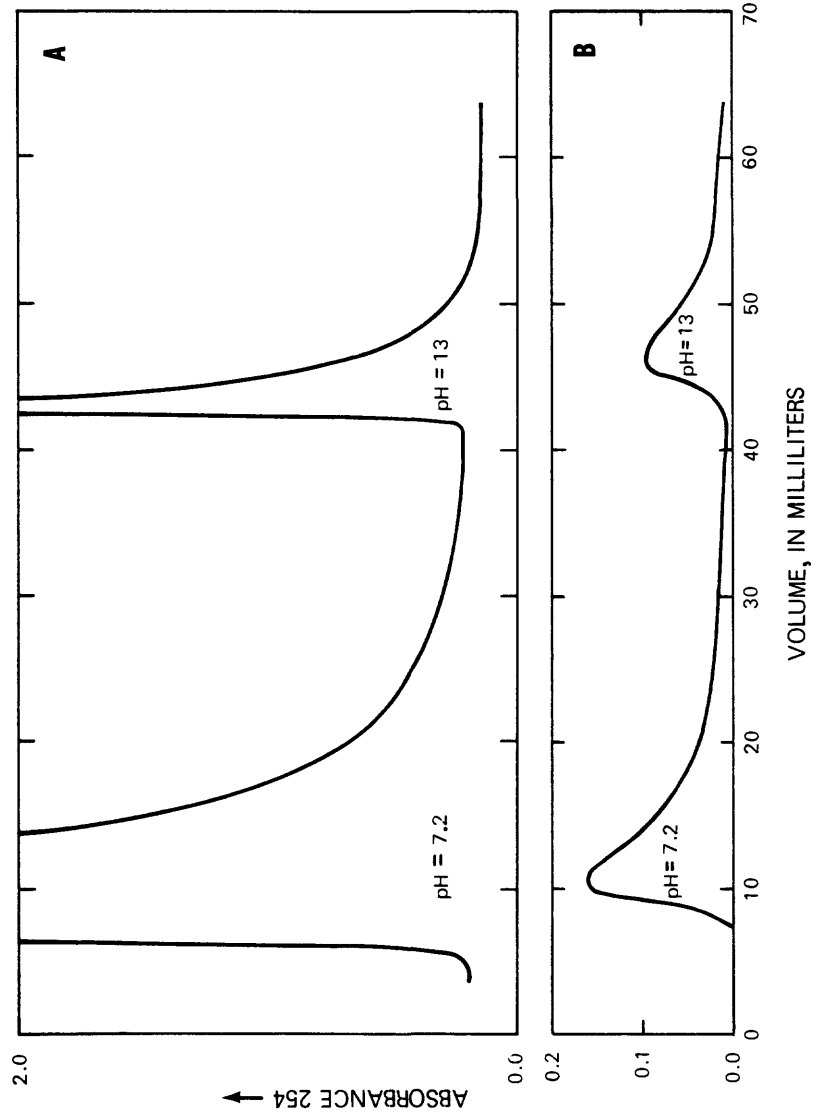

FIGURE 4.—Chromatogram of pH gradient of the (A) high- and (B) low-molecular-weight fractions from the Enzacryl column.

and it may contain compounds such as flavonoids or condensed flavonoids (tannins).

SUMMARY AND CONCLUSIONS

This scheme is useful in separating and concentrating humic substances into a carboxylic acid fraction (fulvic acids), weaker acid fraction (tannic acids), a water fraction, and a methanol fraction. These humic fractions are different with respect to functional group (carboxylic or phenolic hydroxyl) and to the number of functional groups per carbon atom. The scheme allows for the quantification of these various fractions by DOC; now comparisons of humic substances from various water samples can be made. The aqueous fractions appear to be chiefly humic substances. However, the methanol fraction is a mixture of many materials: humic and nonhumic acids as well as neutral compounds. The use of conductivity to detect acid functional groups provides a nondestructive method of estimating the acidic material in this complex fraction.

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