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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF HUMIC ACID IN ENVIRONMENTAL SAMPLES AT THE NANOGRAM LEVEL USING FLUORESCENCE DETECTION[a]

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SUMMARY

A high-performance liquid chromatographic method for the determination of humic acid in environmental samples is presented. The humic acid is chromatographed as its sodium or calcium complex, eluting as a single, sharp peak. Coral skeletal matter, sea water, river water, soils and plant matter were successfully analysed. The detection limit is 15 ng. The relative standard deviation for a coral skeletal sample is 1.9%. Unusual chromatographic properties such as the occurrence of peak broadening with increased concentration appear to be due to a slow change in the equilibrium composition of humic acid. In solution, fulvic acid showed similar properties to humic acid.

INTRODUCTION

Humic acids are ubiquitous, fluorescent organic compounds that possess acidic properties and readily complex with metal ions and organics. They have been detected in soil[1], sediment[2], lake water[2,3] and sea water[4]. Commercial preparations are commonly obtained from coal beds. Even though the first comprehensive studies of their chemical properties were begun over three decades ago, there is still no agreement on many of their fundamental properties[5,6]. Reports of molecular weight, elemental composition and the nature and levels of moieties vary widely[7–12]. The problem has been contributed to, at least in part, by the poor definition of humic and fulvic acids. Humic acid is regarded as the alkali-soluble extract from soils subjected to varying, but undefined, methods of purification. Fulvic acid is the acid-soluble fraction of these extracts. No definitive measure of purity has been set and "humic acid" extracts no doubt are mixtures of organic compounds derived from plant matter[13].

Humic acid influences many diverse activities and processes[14–17]. The impetus for this study was the discovery that massive corals contain fluorescing bands of fulvic (humic) acid within their skeletal structure[18]. The intensity of these bands is

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highly correlated with nearby river discharge volumes. This leads to the potential reconstruction of river discharge and rainfall levels[19]. To study and determine the low concentrations of humic acid in coral skeletal matter and to establish its source requires a sensitive and accurate analytical technique. Given the almost routine use of high-performance liquid chromatography (HPLC) for the separation, quantification and purification of a wide variety of organic compounds in recent years, it is surprising that so few HPLC studies of humic acid have been reported. Those that have appeared in the literature are qualitative rather than quantitative[12,20–24].

Our studies show that humic acid can be readily determined on a glass-lined reversed-phase column as the sodium or calcium complex. Although a reversed-phase column was chosen, the retention mechanism is not known. Fluorimetric detection was used, achieving a detection limit at the nanogram level. Humic acid concentrations in coral skeletal matter, sea water, river water, soils and plant matter have been successfully determined.

EXPERIMENTAL

Apparatus

The HPLC system consisted of a Waters Assoc. (Milford, MA, U.S.A.) M6000 pump, U6K injector and 440 UV detector, a Schoeffel Instruments (Westwood, NJ, U.S.A.) FS 970 fluorometer and a Hewlett-Packard (Palo Alto, CA, U.S.A.) 3392A computing integrator. Wherever possible, PTFE capillary tubing obtained from SGE (Ringwood, Victoria, Australia) was used for the chromatographic lines. Access to a Hewlett-Packard 1090M HPLC system with an inbuilt diode-array variable absorbance detector was obtained. A Varian SuperScan 3 absorbance spectrophotometer (Mulgrave, Victoria, Australia) and a Hitachi (Tokyo, Japan) F-4000 fluorescence spectrophotometer were used for samples not requiring fractionation. The Hitachi spectrophotometer was also used to obtain fluorescence spectra of HPLC-purified humic acids by using the flow cell attachment. A Perkin-Elmer 1640 Fourier transform infrared spectrophotometer with disc drive was used to obtain infrared scans.

Columns

Commercial, pre-packed analytical HPLC columns were purchased. All had a particle size of 5 μm except where indicated below. Guard columns contained the same packing as the analytical columns and were used where indicated. An Upchurch Scientific 2-μm in-line precolumn filter (Oak Harbor, WA, U.S.A.) was routinely used to protect the column from sample particulate matter. The following columns were used: SGE ODS, glass-lined (250 × 4 mm I.D.), plus guard column; SGE SAX, glass-lined (250 × 4 mm I.D.); Brownlee (Santa Clara, CA, U.S.A.) RP-18 Spheri-10 (250 × 4.6 mm I.D.), 10-μm particle size (silanized), plus guard column; Brownlee Phenyl Spheri-5 (100 × 4.6 mm I.D.); Brownlee Amino Spheri-5 (100 × 4.6 mm I.D.), plus guard column; Applied Science (State College, PA, U.S.A.) Cyano Spherisorb (250 × 4.6 mm I.D.); Merck (Darmstadt, F.R.G.) 100 Diol LiChrospher (250 × 4.6 mm I.D.), 100 Å pore size, plus guard column; and Merck 100 CH-18/2 LiChrospher (100 × 4.6 mm I.D.), 100 Å pore size.

Chemicals and solvents

Technical-grade humic acid as the sodium salt was purchased from Aldrich (Gillingham, U.K.). Water was purified with a Millipore (Bedford, MA, U.S.A.) purification system. This Super-Q water was further purified by double distillation from potassium permanganate. Analytical-reagent grade sodium chloride from Ajax Chemicals (Auburn, N.S.W., Australia) was heated at 550°C for 16 h to remove humic acid impurities. Concentrated hydrochloric acid and sodium hydroxide were of Aristar grade, purchased from BDH Chemicals (Poole, U.K.). Tetrahydroxy-*p*-benzoquinone, chloranilic acid, arbutin, quercitin, rutin, alizarin, quinalizarin, lawsone, juglone, hydroquinone and *p*-benzoquinone were purchased from Sigma (St. Louis, MO, U.S.A.). All other chemicals were of analytical-reagent grade.

HPLC-grade methanol was obtained from BDH Chemicals and HPLC-grade tetrahydrofuran and acetonitrile from Waters Assoc. These solvents were mixed in varying proportions with Super-Q water. Ammonia from Ajax Chemicals was added to water at a concentration of 0.003% (w/v) (as NH_3) to give the mobile phase that was used in routine analysis. All buffer and salt solutions were prepared by dissolving the appropriate compounds in water.

Humic acid (Aldrich) was purified by mixing it with Super-Q water, adding ammonia solution to adjust the pH to about 8 and carefully decanting the dissolved portion. This solution was precipitated by the addition of concentrated hydrochloric acid, filtered, air-dried at 22°C for 24 h and heat-dried at 80°C for 16 h. The procedure was repeated twice. The low nitrogen content of 0.83% (w/w) indicated that ammonia had not condensed with humic acid.

Sample preparation

The development of the sample preparation procedures for waters and coral skeletal extract are described in the main text. For soils, approximately 50 mg of sample that had been air-dried at ambient temperature for 2 weeks were placed in a 4-ml glass vial and extracted with 2.00 ml of 0.5 *M* sodium chloride–0.3% (w/v) ammonia solution. The sample was manually shaken for 2 min, then sonicated for 1 h. This was left standing at 4°C overnight to allow the particulate matter to settle. The supernatant was withdrawn (20–200 μl) and placed in a 4-ml glass vial. Sodium chloride solution (5 *M*) was added to bring the final sodium chloride concentration to 0.5 *M*. The final volume was brought to 500 μl by the addition of water. Living and senescent leaves of local grasses, trees and shrubs were manually cut into segments of about 1 mm square and freeze-dried for 40 h. Approximately 10 mg were weighed into a 4-ml glass vial. The plant matter was then extracted by the same method that was used for the soil samples, including the dilution step. When local blue-green algal blooms occurred, sea water that contained these organisms was collected. The sample was frozen at −18°C and allowed to thaw at ambient temperature. This ruptured the cells, allowing the organics to leach into the water. An appropriate aliquot was diluted with water and adjusted to a final sodium chloride concentration of 0.5 *M*. All standards were prepared by adding purified humic acid (Aldrich) to a sample matrix. A calibration graph was constructed and used for quantitation. The injection volume was 450 μl for sea water, 100 μl for coral skeletal and river water samples and 50 μl for soil and plant matter extracts.

Dried grasses were extracted with water and the extract was acidified with

concentrated hydrochloric acid to pH <1. The precipitate was filtered, dried and dissolved in dilute sodium hydroxide solution to a final pH of 8. Excess calcium chloride was added and the resulting precipitate was filtered and dried at 80°C. The calcium complex of humic acid was obtained in a similar manner. These samples were used for infrared analysis (potassium bromide disc).

RESULTS AND DISCUSSION

The chromatography of humic acid has been reported using both classical and high-performance techniques. Size-exclusion columns have been most commonly used. When salt solutions were used as the mobile phase, the peak shapes were usually broad and ill-defined. In one study it was shown that the retention time and peak shape of humic acid varied with the ionic strength of the mobile phase[22]. Sharp peaks were obtained when water alone was used as the mobile phase[20–22].

Diol-bonded silica column

We decided to begin our studies with a diol-bonded silica (diol) size-exclusion column. It was expected that our results could then be compared with those of previous studies in which both classical and HPLC size-exclusion columns were used. When phosphate and acetate buffers and sodium chloride solutions were used at concentrations between 0.001 and 2 *M* and the humic acid concentration was between 8 and 50 μg/ml, a non-reproducible broad band eluted. In contrast, when water was used as the mobile phase, a sharp, single peak eluted close to the solvent front. However, with increasing concentrations of humic acid, the peak broadened, although remaining symmetrical. The eluting peak was collected in three equal fractions and re-chromatographed. Each fraction eluted at the average retention time of the original fraction and the peak shape was again symmetrical. The fluorescence (excitation 340 nm; emission >490 nm) to absorbance (340 nm) ratio successively decreased for the three fractions. On the addition of sodium chloride or calcium chloride to each fraction, a single, sharp peak eluted at an increased retention time that was identical for each fraction. Typical chromatograms for a diol column with acetate buffer and water as the mobile phase are shown in Fig. 1.

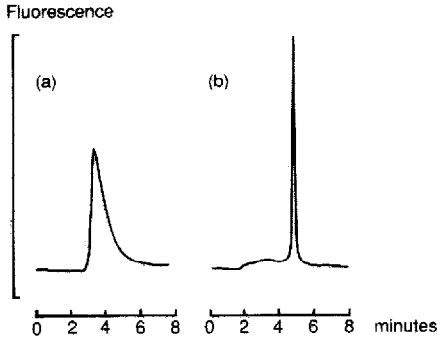


Fig. 1. Peak shape resulting from 1 μg of humic acid injected onto a diol column. Mobile phase: (a) 0.1 *M* sodium acetate solution, pH 6.8; (b) water.

Further unusual properties were observed on investigating the behaviour of humic acid on a Waters Assoc. ODS Sep-Pak cartridge. The retention and elution rate were highly dependent on the humic acid concentration and on the rate and frequency of flushing with water. If the column was allowed to stand for several minutes after a clear eluate was obtained, on renewed flushing a concentrated humic acid solution eluted. This phenomenon could be reproduced by using water, acetonitrile, methanol and tetrahydrofuran in any consecutive elution order.

These characteristics indicated that humic acid in solution was a mixture of components in equilibrium. One component is strongly retained on a reversed-phase column and the other is poorly retained, probably reflecting polarity differences. When disturbed, the equilibrium requires up to several minutes to re-establish, *i.e.*, it is a slow equilibrium. This in turn suggested that the HPLC peak broadening effect was not due exclusively to a size-exclusion mechanism. A slow change in the equilibrium composition of the humic acid could also account for the peak broadening. With water as the mobile phase, the mechanism would almost certainly not be size exclusion. It has been demonstrated previously that size-exclusion columns do not necessarily function in a size-exclusion manner[25].

Other bonded silica columns

When water was used as the mobile phase and < 1 μg of Aldrich humic acid was injected, a single, sharp peak with a low retention time eluted from octyldecylsilyl (ODS), phenyl-bonded silica (phenyl) and cyano-bonded silica (cyano) columns. No peaks eluted from an amino-bonded silica (amino) column or a strong anion-exchange-bonded silica (SAX) column. With phosphate and acetate buffers and sodium chloride solutions in the concentration range 0.001–2 *M*, and with water acidified with perchloric acid to below pH 5, no peaks eluted from an ODS or amino column. The phenyl and cyano columns gave broad, non-reproducible bands with these mobile phases.

Cation complexation

When increasing concentrations of ammonium chloride, sodium chloride, magnesium sulphate and calcium chloride in the range 0.05–1 *M* were added to a standard humic acid (Aldrich) solution (0.02 mg/ml) and chromatographed on an ODS col-

TABLE I

RETENTION TIME AND RELATIVE FLUORESCENCE (EXCITATION 340 nm; EMISSION >418 nm) OF THE FREE AND CATION-COMPLEXED HUMIC ACID ELUTING FROM A GLASS-LINED ODS COLUMN

Solvent flow-rate: 1 ml/min.

Species	*Cation concentration (M)*	*Retention time (min)*	*Relative fluorescence*
Free acid	–	1.36	1.00
Ammonium	0.5	2.10	0.61
Sodium	0.5	2.10	0.91
Magnesium	0.25	2.08	0.63
Calcium	0.2	2.09	0.38

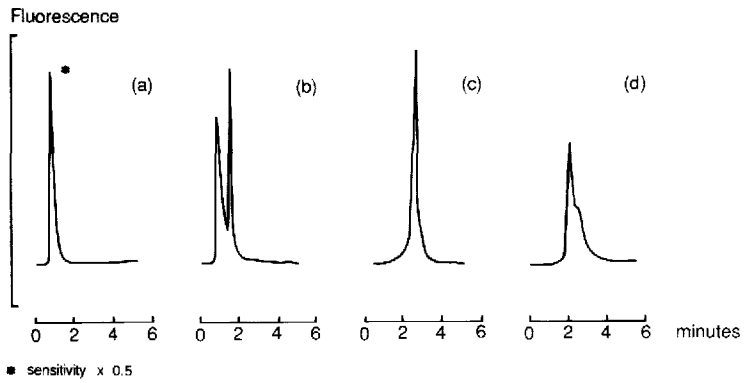


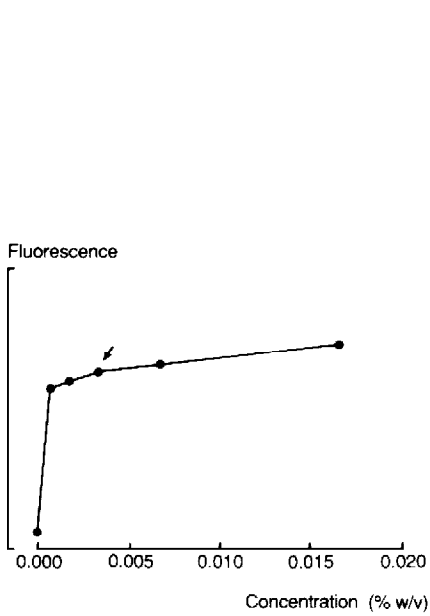
Fig. 2. Succession of peaks obtained when an increasing concentration of sodium chloride is added to 0.02 mg/ml of humic acid and the solution is injected onto an ODS column. Sodium chloride concentration: (a) 0; (b) 0.05; (c) 0.5; (d) 3 *M*.

umn, new peaks and shoulders appeared. The retention times of the existing peaks also increased slightly. The successive chromatograms that were obtained with sodium chloride are shown in Fig. 2. The relative fluorescence of the humic acid peaks resulting from the addition of these salts is given in Table I. Humic acid chromatographed on the diol, cyano and phenyl columns also exhibited similar properties.

In a previous study[2] using paper chromatography, it was recognized that the multiplicity of bands obtained was due to a humic acid–salt interaction. This led to the description of a humic acid–salt interaction hypothesis, but it did not gain general acceptance. When we added calcium chloride to a humic acid solution (2 mg/ml), a precipitate formed immediately and was filtered, washed and dried. It was insoluble in 0.1 *M* sodium hydroxide solution, an excellent solvent for humic acid, but it was soluble in a 0.1 *M* sodium hydroxide–1% (w/v) EDTA solution. This, and the fact that the compound had a 6.3% (w/w) calcium content (by direct current plasma emission analysis), indicated that the humic acid had formed a complex with calcium. When magnesium sulphate was added to the humic acid, a precipitate formed within a few minutes. With sodium chloride and ammonium chloride, a precipitate did not form for several hours. With a more dilute humic acid solution (0.2 mg/ml), no precipitate occurred with any of these salts, but the humic acid complex was strongly retained on an ODS Sep-Pak cartridge. Humic acids are known to complex with metal[26], calcium and hydrogen ions[27]. When sodium chloride and sodium perchlorate were added to the humic acid and subjected to HPLC, a new peak eluted. A new peak did not elute when sodium dodecyl sulphate was added. This indicates that humic acid can complex with sodium ions only in the presence of anions that are poor counter-complexing agents. Our HPLC data indicate that humic acid forms complexes with ammonium and sodium ions. This is in addition to the well known complexes formed with metals such as iron, copper and calcium. As the retention times were increased and the fluorescence was maintained by the addition of salts such as sodium chloride and calcium chloride, all samples and standards were chromatographed as their complexes. Coral skeletal matter was run as the calcium complex and all other samples as their sodium complexes.

Recovery from a reversed-phase column

When samples were injected consecutively (onto any column from which humic acid eluted) within 5 min of each other, a disturbing feature was that the peak size decreased continuously until the peak could not be distinguished from the baseline noise. This generally occurred within about ten injections and was observed for both the free and complexed humic acid. This type of phenomenon had been observed previously with bilins and was overcome in that instance by silylating the ODS column packing material[28]. This approach proved unsuccessful with humic acid, indicating that the problem was not due to irreversible adsorption onto free silanol groups. An increase in the pH of the mobile phase by the addition of ammonia overcame the problem. The elution profile of humic acid against ammonia concentration is shown in Fig. 3. When different columns were used it was found that the humic acid recovery varied considerably. A number of columns were compared using 0.003% (w/v) ammonia solution as the mobile phase. Results are shown in Fig. 4. Two glass-lined ODS columns gave the highest recovery, with similar absolute values. This indicates that humic acid interacts with the stainless-steel surfaces of the columns. Only glass-lined columns are considered suitable for quantitative analysis. To minimize the interaction with other metal components of the HPLC system, capillary PTFE tubing was used where possible. A mobile phase consisting of 0.003% ammonia solution was chosen as a compromise between humic acid recovery and solution pH. This mobile phase, which had a pH of 7.5, was pumped through a single ODS



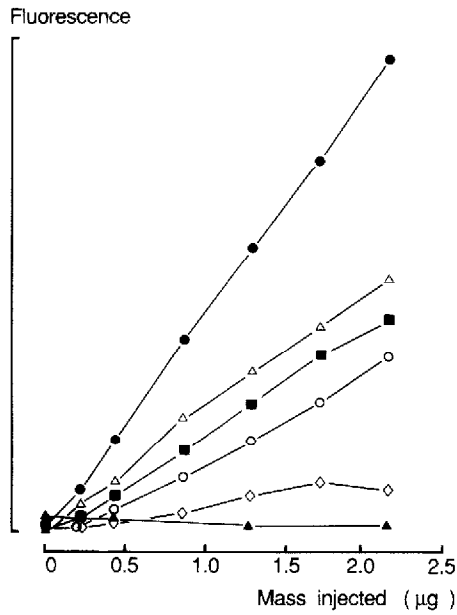


Fig. 3. Recovery of 0.2 μg of humic acid injected onto a glass-lined ODS column as a function of ammonia concentration in the mobile phase. The ammonia concentration used in the analytical procedure is indicated with an arrow.

Fig. 4. Relative recoveries for different amounts of humic acid injected onto the following columns: ● = glass-lined ODS; △ = diol; ■ = phenyl; ○ = ODS; ◇ = cyano; ▲ = amino.

glass-lined analytical column protected by a guard column for a period in excess of 500 h. If the guard column was replaced when the peak shape began to deteriorate, there was no discernible change in the chromatographic properties of the system.

The absolute recovery from a glass-lined ODS column was checked by injecting a humic acid standard (Aldrich) with and without the analytical column. With fluorescence detection (excitation 340 nm; emission >418 nm), the recovery was 85.3%. This must be considered as a minimum recovery as it is possible that other components in the standard were not eluted. The method cannot achieve quantitative recovery because of the pH restraint that we imposed to prolong the column life, but the recoveries were found to be consistent. When river and sea water samples were spiked with a standard humic acid solution at a concentration of 0.02 mg/ml, the peak area for these samples (with the blank subtracted) was the same as for a standard humic acid solution in water at this concentration. This indicates that river and sea water do not adversely affect the humic acid recovery. Consecutive injections of humic acid samples dissolved in 0.5 *M* sodium chloride and in 0.25 *M* calcium chloride solutions were made. After the first two injections, no changes in peak area and height could be observed. In the analytical procedure, the column was routinely equilibrated with two 50-μl injections of a 0.2 mg/ml standard humic acid solution.

Fluorescence detection rather than UV absorbance detection was chosen because it showed better sensitivity and selectivity. Absorbance detection was not investigated in any detail, although preliminary studies indicated that absorbance monitoring at 254 nm may give inconsistent results.

The effect of organic modifiers with an ODS column was determined. Methanol, tetrahydrofuran and acetonitrile were mixed with water at concentrations between 5% and 80% (v/v). Little change in retention time was observed, even for high concentrations of the organic solvent. However, with increasing organic solvent concentration, the peak shape deteriorated until the peak could not be distinguished from the baseline noise. The peak shape was also sensitive to the amount of humic acid injected. When 10 μg of humic acid (Aldrich) was injected, a broad band with multiple sub-peaks eluted, similar to the elution profile in a previous study[24]. A comparison of retention mechanisms with that study cannot be made because different mobile phases were used. However, our data indicate that a reversed-phase mechanism is precluded. The equilibrium composition described above may have a significant influence on the mechanism.

Humic acid fluorescence and linearity

The fluorescence excitation spectra of humic acid (Aldrich and environmental samples) solutions changed markedly between concentrations of 0.5 and 140 μg/ml, although the emission spectra remained similar. This phenomenon could be due to a change in the equilibrium composition that we suggested above. Fluorescence at various excitation wavelengths over this concentration range for Aldrich humic acid was measured. Linearity was only obtained at the lower concentrations. The linear range decreased with decreasing excitation wavelength. Because our HPLC detector was fitted with a deuterium lamp, an excitation wavelength of 340 nm was chosen. At this wavelength, a good signal-to-noise ratio could be obtained and initial measurements indicated that linearity could be achieved at normal HPLC concentrations. Emission was measured at >418 nm with the aid of a cut-off filter. With these

wavelength settings, linearity was determined for the HPLC system using coral skeletal, sea water, river water, soil and plant matter samples. Humic acid was added to the sample matrix and the final sodium chloride and calcium chloride concentrations were adjusted to 0.5 and 0.2 *M*, respectively. For a 50-μl injection, linearity was maintained between 0.18 and 2.1 μg of humic acid injected ($r^2 > 0.997$). Coral skeletal, sea water and river water samples did not require dilution owing to their low levels of humic acid. Soil and plant matter extracts required dilution. Above about 3 μg of humic acid injected, peak-broadening effects can begin to occur. Excitation wavelengths below 340 nm can be used for increased sensitivity, but the linear range is diminished. The fluorescence response was also matrix dependent, which necessitated the construction of a calibration graph for each sample matrix.

Spectral properties of the HPLC peaks

The nature of the multiple peaks originating from the successive addition of ammonium, sodium, magnesium and calcium ions was investigated. When the concentrations of ammonium chloride and sodium chloride were about 0.5 *M*, and the concentrations of magnesium sulphate and calcium chloride were about 0.25 *M*, a sharp, single peak eluted from an ODS column with only a minor leading edge but a clearly distinguishable trailing edge at a flow-rate of 0.2 ml/min. The exact concentration that gave a single peak varied from column to column and had to be determined prior to routine analysis. For two glass-lined ODS columns tested, sea water samples could be injected directly without adjustment of the sodium chloride concentration.

Humic acid (Aldrich) was dissolved in 0.5 *M* sodium chloride and injected into an HPLC system fitted with a diode-array detector. Absorbance scans of the eluting peak were taken at millisecond intervals. An HPLC trace and a comparison of the spectra at six points along the peak are shown in Fig. 5. Between 220 and 400 nm the spectral differences were negligible. When the sample was prepared without sodium chloride, the individual spectra were again very similar to each other and also to those obtained with added sodium chloride. These results are similar to those obtained in a previous study[24] and confirm that the humic acid peak does not consist of a mixture of many different components. With an ODS column, two distinct peaks and a shoul-

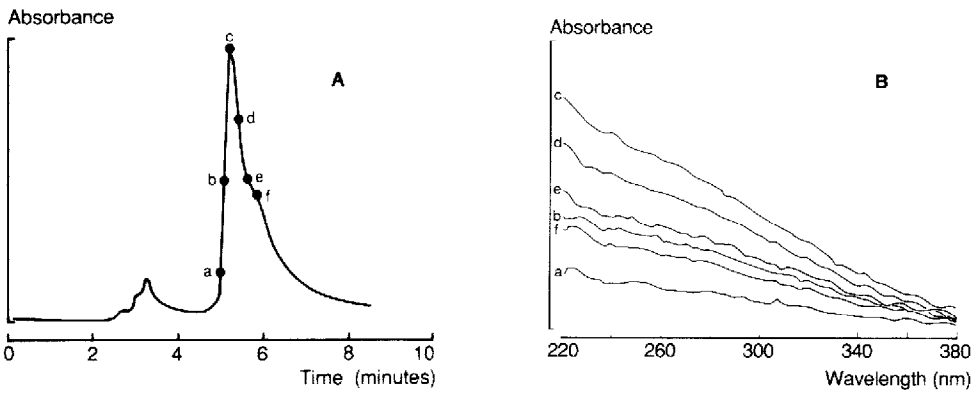


Fig. 5. (A) HPLC 250-nm absorbance trace with (B) spectra from the specified points of an eluting humic acid peak. The absorbance spectra were recorded with a diode-array detector during elution.

der eluted as the sodium chloride or calcium chloride concentration in the sample was increased from 0 to 2 *M*. These peaks are due to the free acid and probably to two cationic complexes. It has been observed previously that humic acid has two binding sites for calcium and hydrogen ions[27]. Sodium ions may complex at the same sites.

Interfering substances

To check for interfering substances, a number of acidic and polar compounds similar to compounds of plant origin were chromatographed, including lawsone, juglone, tetrahydroxy-*p*-benzoquinone, chloranilic acid, hydroquinone, *p*-benzoquinone, quercetin, rutin, arbutin, alizarin and quinalizarin. Chloranilic acid, quercetin and tetrahydroxy-*p*-benzoquinone eluted unresolved from humic acid on a glass-lined ODS column with 254-nm absorbance detection. When dissolved in 0.5 *M* sodium chloride they were also not resolved, coeluting with humic acid. Lawsone coeluted with the sodium complex of humic acid. As only humic acid was fluorescent, however, these compounds did not interfere when fluorescence detection was used.

To guard against possible interfering components in actual samples, the fluorescence ratio for an excitation wavelength of 340 nm relative to 270 nm was routinely monitored. For all humic acid samples tested to date, the ratio rarely varied beyond the limits of 0.25 $\pm$ 0.05. This is the ratio that is obtained for a standard Aldrich humic acid, indicating that interfering components are not a problem. The ratio for the peak coeluting with humic acid that was obtained from plant matter was sensitive to the extraction method. A ratio as low as 0.29 was only achieved when 5 ml of extracting solvent were used for about 5 mg of dry plant matter. If the proportion of plant matter was greater than this, the ratio was always higher. When water alone was used to extract the plant matter, many peaks occurred, but they fused into one peak on dilution plus the addition of sodium chloride. On the addition of amino acids and proteins to a standard humic acid solution, new peaks also occurred. The organic compound extracted from plant matter by water was probably complexed with other organic compounds such as amino acids. These organics could simply be replaced by diluting the solution and adding sodium chloride. Direct extraction with a 0.5 *M* sodium chloride–0.3% ammonia solution also overcame the problem.

Application of the method

The supernatant from the acid precipitation of Aldrich humic acid (fulvic acid by definition) was siphoned-off and freeze-dried. The resulting sample was dissolved in water and chromatographed as the free acid and as the sodium complex. The retention times were identical to those obtained for humic acid. The fluorescence ratios for an excitation wavelength of 340 nm relative to 270 nm were 0.25 and 0.26 for the humic and fulvic acids, respectively. In an analogous experiment, the supernatant from a calcium chloride precipitation experiment was chromatographed. The retention time showed that the soluble humic acid fraction was also complexed with calcium. These data indicate that, in solution, humic acid and fulvic acid are very similar.

The HPLC-purified humic acid that remained in the flow cell after diversion of the mobile phase was scanned for fluorescence excitation (240–380 nm; emission 440 nm) and emission (400–600 nm; excitation 340 nm) spectra. The spectra of the Aldrich humic acid were almost identical with those of coral skeletal, sea water, river

water, soil and plant matter samples. At least four different sources of each sample were chromatographed and scanned to confirm this. Because the excitation spectra change with concentration as noted above, samples were prepared to give approximately equal fluorescence responses (excitation 340 nm; emission 440 nm). A surprising result was obtained from plant leaf extracts. Both the retention time of the HPLC peak and its fluorescence excitation and emission spectra were similar to that obtained for humic acid from soils. The calcium complexes of Aldrich humic acid and of a dried grass extract showed almost identical infrared spectra in the region 4000–500 cm^{-1}. These data indicate that plants are a direct source of humic acid, in addition to the generally accepted indirect source. Fulvic acids have been isolated from leaf leachates[29]. For the extract from the algal bloom, there was a small difference in the 280–340-nm region of the fluorescence excitation spectrum compared with the Aldrich humic acid. The emission spectra were similar.

TABLE II

COMPARISON OF HUMIC ACID CONCENTRATIONS OBTAINED BY DIRECT FLUORESCENCE (EXCITATION 340 nm; EMISSION 440 nm) AND HPLC MEASUREMENTS

Sample	*HPLC*	*Direct fluorescence*
Coral skeletal[a] (μg/g)	57.7	116.9
	27.5	71.1
	25.2	51.6
	34.6	78.3
	44.9	85.4
	24.1	77.5
	29.4	62.7
	43.7	84.7
	19.8	38.6
	37.1	78.7
Inshore sea water[b,c] (μg/ml)	0.72	0.71
	0.42	0.47
	0.77	0.90
	0.81	0.81
Offshore sea water[b,d] (μg/ml)	0.11	0.53
	0.09	0.41
	0.08	0.30
	0.11	0.34
River water[b] (μg/ml)	9.10	9.56
	7.94	7.16
	1.79	1.88
	1.41	1.58
Soil[b] (μg/g)	1280	2260
	2060	3920
	711	1470
	5420	8560

[a] Ten samples from a single source.
[b] Four different sources.
[c] Within 500 m of the shore.
[d] 40–90 km offshore.

The chromatographic and fluorescence data indicate that the Aldrich humic acid is similar to humic acids obtained from coral skeletons, sea water, river water, soil and plant leaves, after purification by HPLC. Aldrich humic acid can therefore be used as a standard to determine humic acid in environmental samples. Typical humic acid concentrations in coral skeletons, sea water, river water and soils are given in Table II. The results of the HPLC method are compared with results obtained by measuring the fluorescence levels of the unchromatographed sample. The excitation (240–380 nm; emission 440 nm) and emission (400–600 nm; excitation 340 nm) spectra of unchromatographed coral skeletal, offshore sea water and soil samples were different to those of humic acid, indicating the presence of interfering fluorescent compounds. For these samples, HPLC analysis gave lower humic acid concentrations than direct fluorescence measurements. Direct fluorescence and HPLC measurements gave comparable results for river water and inshore sea water, indicating that humic acid is the major contributor to the fluorescence of these samples. The desirability of determining humic acid by chromatography is indicated by these data.

TABLE III

SUMMARY OF CHARACTERISTICS OF THE HPLC METHOD

Column	Glass-lined ODS (250 × 4.6 mm I.D.)
Mobile phase	0.003% (w/v) ammonia solution
Flow-rate	1 ml/min
Retention time	2.1 min (sodium complex)
Injection volume	50–450 μl
Detection	Fluorescence (excitation 340 nm; emission >418 nm)
Detection limit	15 ng (2 × noise)[a]
Relative standard deviation	(i) 1.9% (n = 8)[b]
	(ii) 0.34% (n = 10)[c]

[a] A detection limit of 1.3 ng was achieved for a sodium complex excited at 255 nm and run on a diol column.

[b] For a coral skeletal sample.

[c] For a river water sample excited at 270 nm.

CONCLUSION

Full details and characteristics of the method are given in Table III. At present the retention mechanism of humic acid on HPLC columns is not known, but the use of an appropriate mobile phase elutes a single, sharp peak from various columns. On an ODS column, humic acid is well resolved from many other polar and acidic compounds, and with fluorescence detection interfering compounds have not been observed. Investigations are in progress to develop a technique that will fractionate the individual components of a purified humic acid. The method described in this paper has been used routinely for the separation and determination of humic acid from many environmental sources. Humic acid environmental levels, their significance and their incorporation into coral skeletal matter will be the subject of a further report.

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