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Analysis of fulvic acids by ion-pair chromatography

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ABSTRACT

Ion-pair chromatography, using tetrabutyl ammonium hydroxide and a reversed-phase column, has been applied to the analysis of groundwater in order to demonstrate the usefulness of the technique in the investigation of dissolved organic matter. Results show that fulvic acid, present in groundwater, can be separated into a number of naturally occurring organic constituents. Chromatograms obtained from fulvic acid that was extracted from the groundwater using DEAE cellulose were very similar to those obtained from the groundwater. Ion-pair chromatography is therefore useful for investigating the components that constitute fulvic acid which may be present in groundwater or extracted from the groundwater. Metal complexation studies can also be investigated by ion-pair chromatography.

INTRODUCTION

Fulvic acids are a heterogeneous class of naturally occurring compounds which constitute a large proportion of dissolved organic matter in groundwater. They are important because of their ability to bind with and mobilise metal species and anthropogenic organic species [1,2]. The heterogeneous nature of these compounds means that, even within a sample of fulvic acid, there is a distribution of molecular size and charge [3,4]. Studies on these distributions, and their importance to (say) metal binding have been hindered by a lack of suitable analytical techniques. Chromatographic separations of components within a fulvic acid sample have been attempted and these have given some insight into the importance of the components that make up fulvic acid. Size-exclusion chromatography has been extensively used to investigate the size distribution of the molecules [5–7] but, this technique gives little information on the components within a fulvic acid sample. High-performance ion chromatography has been used to separate many naturally occurring organic molecules, but its application to fulvic acid analysis is severely limited due to the high affinity of the resin for acidic groups present in the fulvic molecule. Reversed-phase chromatography has been used to analyse fulvic acids using conventional bonded reversed-phase packings and UV and fluorescence detection [8–10]. Reversed-phase chromatography has also been

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used to fractionate a sample of fulvic acid into five molecular weight ranges and to investigate the effect of molecular weight on the hydrophilicity of the fulvic acid [11]. This chromatographic technique has also been attempted using a microcolumn [12]. Combinations of ion-suppression and reversed-phase chromatography using 1% acetic acid in a water–methanol–acetonitrile mixture have been used to fractionate fulvic acids into hydrophobic and hydrophilic fractions. These studies have generally provided chromatograms of poor resolution because, within the pH range of the suppression for silica bonded columns, fulvic acids are highly charged and would also be excluded from the column packing material by steric effects. Recent work on ion-suppression chromatography [8] has shown that better resolution was obtained when using ion-suppression (pH 4.0) and a Novapak column.

This work describes the application of ion-pair chromatography together with a wide pore polymeric reversed-phase column packing and diode array detection for the analysis of naturally occurring high-molecular-weight organics. As far as the authors are aware this is the first reported application of this technique to the analysis of this type of material.

In ion-pair/reversed-phase chromatography a hydrophobic counterion is added to the mobile phase. This counterion dynamically interacts with the solvated acid and stationary phase to produce an ion pair that has significantly more hydrophobic character than that of the original acid. Such reagents have been routinely used in the analysis of low-molecular-weight organic acids [13]. However, a literature search has not revealed their use for the separation and characterisation of humic and fulvic acids. The major advantage of ion-pair chromatography over ion-suppression chromatography is that near neutral pH conditions may be maintained thus limiting the effect of pH changes on both the stationary phase and solvated molecule.

EXPERIMENTAL

Instrumentation

A Waters Model 600E low pressure mixing quaternary solvent delivery system equipped with semi-preparative pump heads was used. All experiments were performed using a 25 cm × 4.6 mm I.D. Polymer Labs. PLRP-S (300 Å) fitted with a PLRP-S reversed-phase guard column. Samples were introduced into the column by a Waters Wisp 712 autoinjector. The eluate was monitored for UV absorbance using a Waters 990 photodiode array detector. This enabled the UV absorbance of the mobile phase to be determined over the wavelength range 200 to 400 nm.

Materials

Mobile phases of water–acetonitrile mixtures were purchased from Romil Chemicals and were far UV HPLC grade. Tetrabutyl ammonium hydroxide in phosphate buffer was purchased from Fison Scientific and was used as the ion-pair reagent in the separations described below. The concentration of the ion pair reagent in HPLC water was 0.005 *M*. All mobile phases were prefiltered (0.45 μm Nylopore) and degassed with helium before use.

Groundwater taken from a shallow sand aquifer at Drigg in Cumbria was filtered through 0.45 μm Nylopore filter before use. The chemical analysis of the groundwater has been reported [14]. The effects of fulvic acid concentration, and

ionic strength of the groundwater, on the separation were investigated by rotary evaporating a sample of the groundwater to ten times concentration at 35°C (× 10 groundwater).

In order to examine the effects of extracting fulvic acids from the groundwater on chromatographic separation, DEAE cellulose was used to extract fulvic acid from the groundwater [15].

METHOD DEVELOPMENT

Isocratic elution

To investigate the usefulness of isocratic elution and to optimize chromatographic conditions for subsequent gradient elution, chromatograms were run with binary mobile phases consisting of 80:20, 60:40, 40:60 and 20:80, acetonitrile–ion pair solution. The mobile phase flow-rate used in these separations was 1 ml/min. A volume of 20 μl of × 10 Drigg groundwater was injected into the column and the eluate monitored by the photodiode array detector. In order to determine the elution time of non-retained material, HPLC water was injected into the column and detected by the resulting refractive index change in the eluate.

The results of these experiments are summarised in Table I which shows that the retention time of the injected material increases as the concentration of acetonitrile in the mobile phase is lowered. This is consistent with a hydrophobic interaction between the stationary phase and the injected material. With a mobile phase consisting of acetonitrile–ion pair reagent (80:20) retention is strong enough to prevent elution of the more non-polar components. The best mobile phase composition to allow isocratic separation of the components of Drigg groundwater appears to lie between acetonitrile–ion pair reagent (40:60) and acetonitrile–ion pair reagent (20:80).

TABLE I

RESULTS OF CHANGES IN MOBILE PHASE COMPOSITION

Mobile phase composition acetonitrile–ion pair (%)	t_R solvent front (min)	t_R sample peaks (min)
80:20	3.2	3.0
60:40	3.2	3.0, 3.4
40:60	3.2	4.4, 6.6, 8.4, 17
20:80	3.2	6.0

Gradient elution

To obtain greater resolution near the solvent front and to elute strongly retained material, gradient elution was used. Best separation of the components in × 10 groundwater was achieved using the mobile phases and the gradient solvent profiles shown in Fig. 1. The chromatogram contains six UV absorbing maxima with retention times of 3.2, 4.58, 5.52, 10.18, 13.36 and 17.62 min, labelled A to F in the figure. Complete resolution of fulvic acids are unlikely because of the intrinsic heterogeneity of such materials [3,4]. The UV absorption spectra of each eluted peak showed that

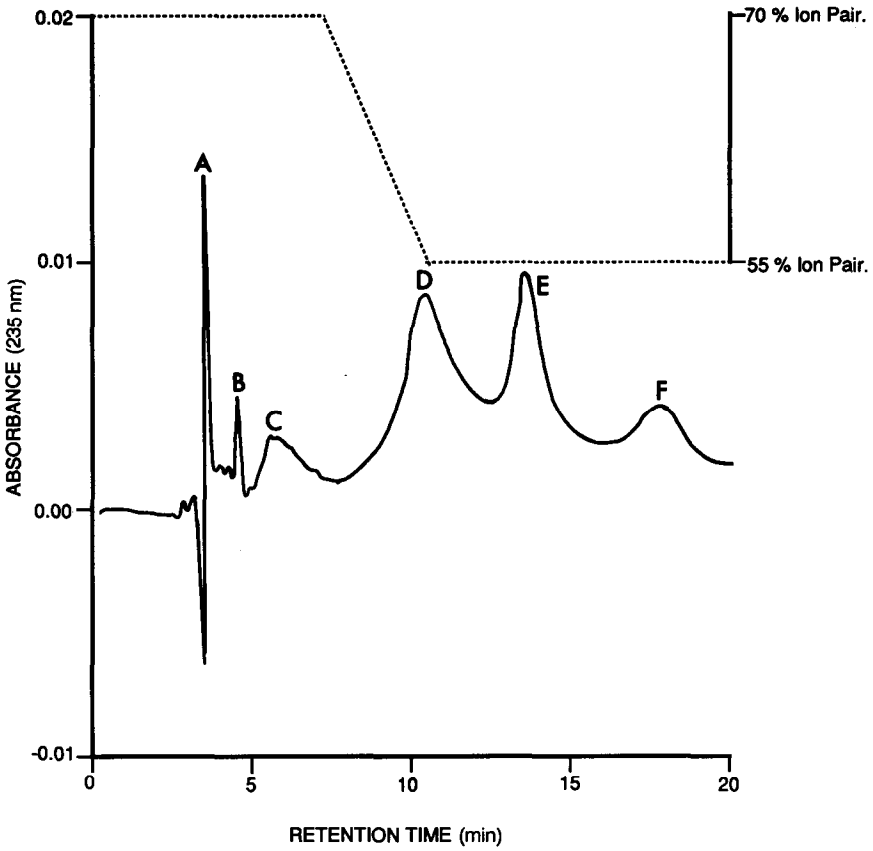

Fig. 1. Chromatogram obtained by the binary separation of × 10 groundwater at a monitoring wavelength of 235 nm.

peaks C, D, E and F were typical of fulvic materials [16] whereas the spectrum observed from peak B was clearly not. To check the reproducibility of the separation, aliquots of the same sample were injected onto the column. The resulting chromatograms were identical.

A volume of 20 μl of HPLC-grade distilled water was injected in to the column in order to observe the UV absorption of the mobile phase as its composition changes with time. The resulting chromatogram showed a peak with a similar retention time to peak A in Fig. 1. This absorption peak is probably due to a refractive index change of the eluate as suggested by its absorption spectrum.

Fig. 2 and 3 are chromatograms obtained by injecting 200 μl of unconcentrated groundwater and 20 μl of extracted fulvic acid in HPLC water (100 μg ml^{-1}). The chromatograms observed from the separations of the groundwater and the extracted material are almost identical to the chromatogram observed from the × 10 groundwater except for the peak (B) at an elution time of 4.58 min. In Fig. 3 this peak is absent. The UV absorption spectrum of this peak indicates that this component is not

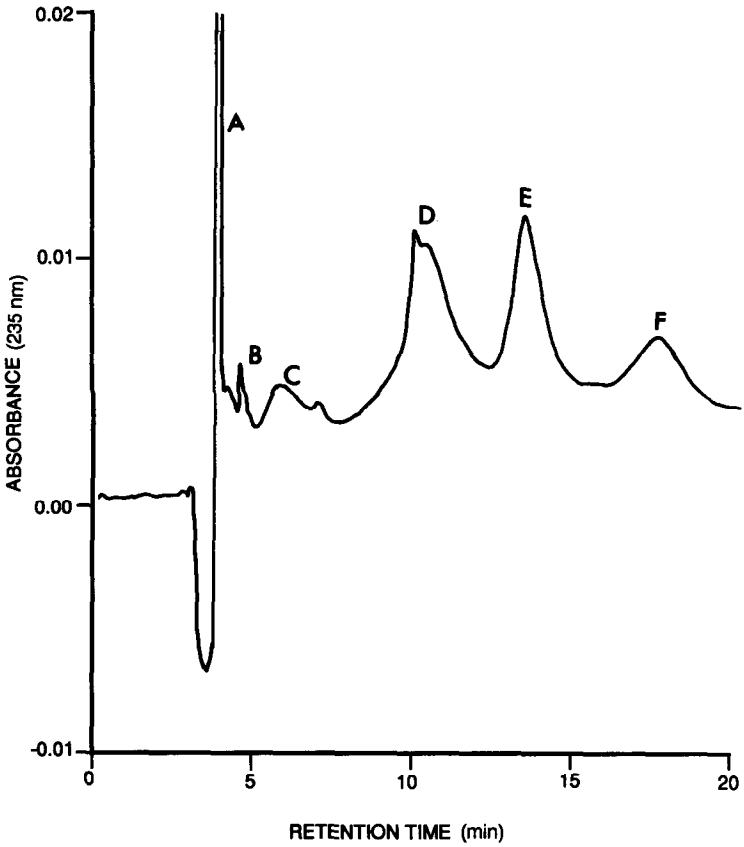

Fig. 2. Chromatogram obtained by the binary gradient separation of 200 μl of unconcentrated groundwater at a monitoring wavelength of 235 nm.

fulvic material and is probably due to the presence of inorganic species in the samples. Further evidence for peak identification was gained from treating × 10 groundwater with DEAE-cellulose and filtering (0.45 μm filter) the treated groundwater before separation on the column. Fig. 4 shows the chromatogram of DEAE treated × 10 groundwater. The separation shows clearly that the fulvic material (peaks C, D, E and F) is extracted from the groundwater, whereas the component which produces peak B is not removed from the water by this treatment. Groundwater was filtered through an Amicon YC05 membrane filter (500 Dalton cut-off) before injecting a sample of the filtrate in to the column. The resulting chromatogram showed the presence of peak B at the same retention time as that observed in Fig. 1. The peaks assigned to Fulvic acids were absent in this chromatogram. The increase in baseline absorbance occurring between 8 and 10 minutes occurs due to changes in the UV absorption of the mobile phase. Superimposed on this increase is a small peak that appears to be due to concentration and subsequent elution of a hydrophobic contaminant present in the mobile phase.

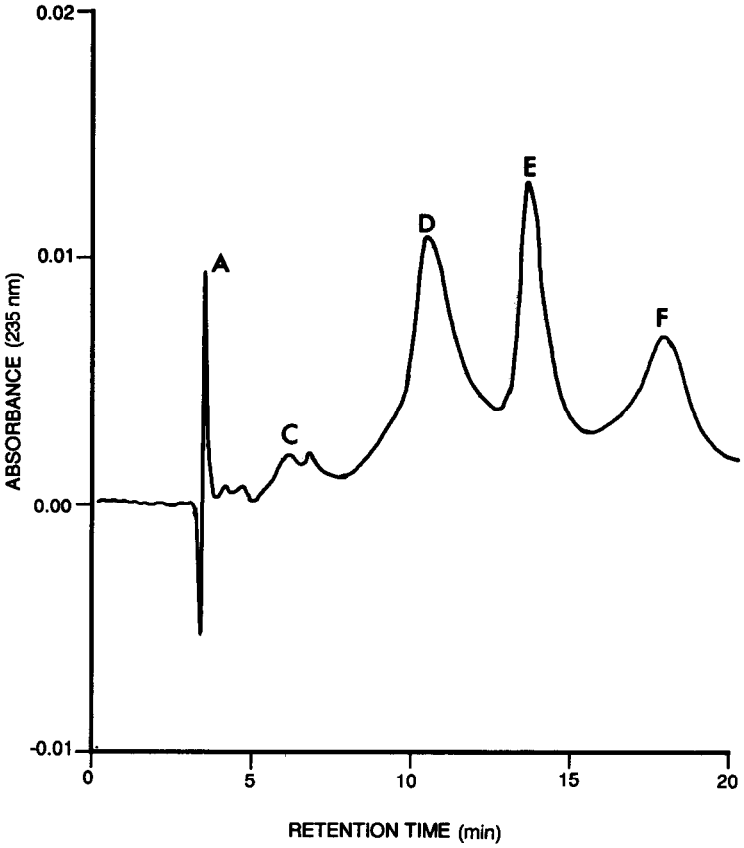

Fig. 3. Chromatogram obtained by the binary gradient separation of 20 μl of extracted fulvic acid in HPLC water (100 μg ml^{-1}) at a monitoring wavelength of 235 nm.

To illustrate the possible use of ion-pair chromatography in metal complexation studies a sample of × 10 groundwater that had been equilibrated with radioactive ^{57}Co was injected onto the column. The eluate was monitored for UV absorbance and fractions were collected at 1-min intervals for radiometric analysis. The results of this study show a close association of eluted ^{57}Co with peaks assigned as fulvic material and suggests that this methodology may be used to study the association of metals with components of fulvic acids without the need to extract the fulvic material from the groundwater.

CONCLUSIONS AND DISCUSSION

Ion-pair chromatography using a large pore stationary phase has been successfully applied to the analysis of fulvic acids in natural and concentrated groundwater samples. The method separates the organic species present in the groundwater into a number of well resolved components, the majority of which have a molecular weight

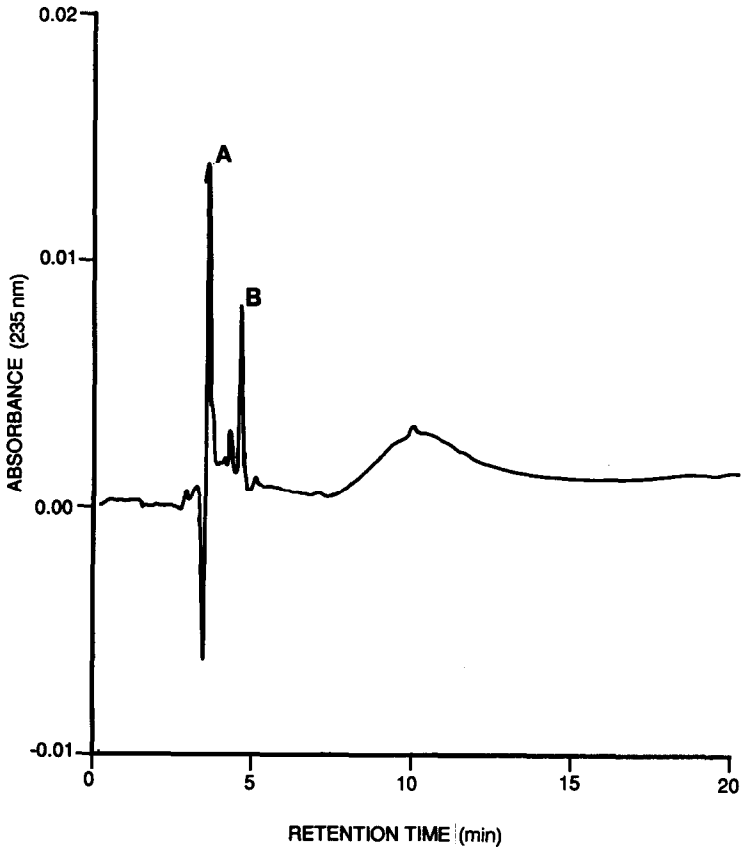

Fig. 4. Chromatogram obtained by the binary gradient separation of 20 μl of DEAE treated × 10 groundwater monitored at a wavelength of 235 nm.

of greater than 500 daltons. Separations obtained have been qualitatively analysed using a diode array spectrophotometer. The components in excess of 500 daltons show UV absorption spectra similar to humic and fulvic acids [16] whereas the component with a molecular weight of less than 500 daltons shows a sharp UV cutoff at 230 nm. It was noted that this component was not removed when the groundwater was passed through DEAE cellulose. The chromatographic separation of × 10 groundwater equilibrated with ^{57}Co has been carried out and results show that Co is associated with fulvic acid present in the groundwater.

Rotary evaporation is an efficient non-invasive method of concentrating the components in groundwater. The large (× 10) difference in the ionic strength of these injected samples does not appear to adversly effect the chromatography, indicating that equilibration of the sample with the mobile phase is rapid.

Further development work is in progress to investigate the use of ion-pair chromatography to study the extraction of organic material from groundwater.

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