Separation of Fulvic Acid from Soil Extracts Based on Ion-Pair Formation with a Cationic Surfactant

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A novel method for separating fulvic acid (FA) from soil extracts is proposed. The FA, defined as the acid-soluble fraction of an alkaline extract of soil, was prepared based on the precipitation of an ion-pair with a cationic surfactant. This method appears to be difficult. However, the flocculation of FA, owing to changing the ionic strength, has been reported. Such a phenomenon, analogous to the salting out of proteins, can be attributed to the fact that anionic charges in FAs are lost. This raises the possibility that FAs might be separated by the precipitation of an ion-pair. If large cationic molecules, such as cationic surfactants, were to be used, large amounts of ion-pair precipitates of FA could be obtained. In the present study, we propose a novel method for the separation of FA from a soil extract based on ion-pair formation with a cationic surfactant. In addition, the chemical characteristics of FA by the proposed method using a cationic surfactant (FA-SUR) were compared with samples prepared by the IHSS method using a DAX resin (FA-DAX).

Introduction

Humic substances (HSs) play important roles in the transformation and transportation of xenobiotics, such as pesticides, in aquatic and soil environments. Fulvic acids (FAs) are defined as the acid-soluble fraction from an alkaline extract of soils, and are known to be water soluble. Thus, elucidating the chemical characteristics of FAs is of crucial importance in understanding the fates of xenobiotics in soil fluids and ground water. In the method of the International Humic Substances Society (IHSS), FAs are separated from the acid supernatant of an alkaline extract of soils using an XAD-8 resin. Since the XAD-8 resin is no longer manufactured, Peuravuori et al. proposed the use of the DAX-8 resin instead of XAD-8.

The separation of FAs using the XAD-8 or DAX-8 resin is mainly based on interactions between hydrophobic moieties in FAs and the resin. Thus, the more hydrophilic fractions of FAs may be lost in the separation step. There have been only a few reports on attempts to retain hydrophilic fractions of FAs. Watanabe and Kuwatsuka reported on the fractionation of FAs with a polyvinylpyrrolidone (PVP) adsorbent, in which the separation was based on hydrogen bonding with phenolic hydroxyl and carboxylic groups in the FAs. In addition, an anion-exchanger, diethylaminoethyl (DEAE) cellulose, was employed for the separation of HSs in aquatic environments, based on electrostatic interactions. Because FAs are known to be soluble at all pH values, the separation of FA based on the formation of a precipitate, as in the case of humic acid (HA), would appear to be difficult. However, the flocculation of FA, owing to changing the ionic strength, has been reported. Such a phenomenon, analogous to the salting out of proteins, can be attributed to the fact that anionic charges in FAs are lost. This raises the possibility that FAs might be separated by the precipitation of an ion-pair. If large cationic molecules, such as cationic surfactants, were to be used, large amounts of ion-pair precipitates of FA could be obtained. In the present study, we proposed a novel method for the separation of FA from a soil extract based on ion-pair formation with a cationic surfactant. In addition, the chemical characteristics of FA by the proposed method using a cationic surfactant (FA-SUR) were compared with samples prepared by the IHSS method using a DAX resin (FA-DAX).

Experimental

Preparation of FAs

IHSS method. A 20 g portion of dry peat soil (Shinshinotsu, Hokkaido, Japan) was extracted with 600 cm³ of aqueous 0.1 M NaOH under an Ar atmosphere, followed by precipitating the HA at pH 1 by the addition of HCl. The FA in the supernatant was adsorbed on a DAX-8 column (Supelco, 60 - 90 µm, 100 mm × 50 mm i.d.) and then eluted with aqueous 0.05 mol dm⁻³ NaOH. The eluted FA fraction was acidified with HCl to pH 4 and then deionized by passing it through a Sephadex G-10 column (Amersham Bioscience, 55 - 165 µm, 100 mm × 50 mm i.d.) using water as the eluent. The FA fraction was then passed through a column of H⁺-type cation-exchange resin (Amberlite, IR-120, 0.3 - 1.1 mm, 100 mm × 50 mm i.d.). The column effluent was lyophilized to give a powdered sample of FA-DAX.
cm³) was neutralized by adding aqueous HCl until the pH of the solution reached 7. In this procedure, some suspended matter was observed and this was separated by centrifugation. The cationic surfactant (2 g, domiphen bromide) was then added to the supernatant. After the mixture was kept for 3 days, the precipitate comprised of ion-pairs between the FA and surfactant was separated by centrifugation, and then dissolved in 30 cm³ of aqueous 0.1 mol dm⁻³ HCl. The surfactant in the aqueous phase was purified by passing it through an H⁺-type cation-exchange column (Amberlite IR-120). After the column effluent was lyophylized, FA-SUR was obtained as a powder.

Optimization of ion-pair formation

A 2 cm³ aliquot of the acidic supernatant was pipetted into a 10 cm³ test tube, and the surfactant (2.5 – 10 mg) was then added. After standing for 6 – 72 h, the precipitate was separated by centrifugation. The FA in the supernatant was analyzed by spectrophotometry at the wavelength of 400 nm. The percentage of FA removal as a precipitate can be calculated as:

\[
\text{% (FA removal)} = \left( \frac{A_{\text{FA}} - A_{\text{FA+SUR}}}{A_{\text{FA}}} \right) \times 100 \quad (1)
\]

where \(A_{\text{FA}}\) and \(A_{\text{FA+SUR}}\) denote the absorbance before and after adding the surfactant, respectively. Four types of cationic surfactants (Fig. 1) were examined: cetyltrimethylammonium bromide (CTAB, FW 364.5); dodecylethyldimethylammonium bromide (DEDAB, FW 322.4); domiphen bromide (DB, FW 414.5); benzyldimethylhexadecylammonium chloride (BDHAC, FW 396.1). These surfactants were purchased from Aldrich and were used without further purification. To optimize the precipitate formation, we investigated the influences of solution pH, type of cationic surfactant, the amounts used and the standing time on the percentage of FA removal.

Analyses of FAs

Elemental compositions (C, H, N, S, and ash contents) of the FAs were determined at the Center for Instrumental Analysis in Hokkaido University (Sapporo, Japan). Total acidity and carboxylic group content were determined by the Ba(OH)₂ and Ca(CH₃COO)₂ methods, respectively. The UV-Vis absorption spectra of FAs were determined using a Jasco V-550 type spectrophotometer. The absorbance values were measured using 50 mg dm⁻³ solutions of FAs in 0.02 mol dm⁻³ citric/phosphate buffer (pH 6), and the absorptivities \((E)\) at 280, 465 and 665 nm were calculated as:

\[
E(\text{cm}^{-1}\text{g}^{-1}\text{of C}) = \frac{\text{absorbance}}{[\text{FA}(\text{g}^{-1})] \times \%\text{C}/100} \quad (2)
\]

\(^1\)H NMR spectra of 0.01 mol dm⁻³ NaOD/D₂O solutions of DB and FA-SUR (1%) were obtained on a JEOL Lambda FT-NMR spectrometer (Nippon Denshi) with a resonance frequency for \(^1\)H of 500 MHz. The solid-state CPMS \(^{13}\)C NMR spectra of DB and FAs were acquired on a Chemagnetics CMX-300 spectrometer, equipped with a 5 mm CPMS probe. A 20 mg portion of powdered sample of FA was packed into a 5-mm zirconium rotor. The acquisition parameters were as follows: spectral frequency, 75.6 MHz for \(^{13}\)C and 300.5 MHz for \(^1\)H; contact time, 1 ms; pulse delay, 4 s; scan times, 20000; line broadening, 50 Hz. The spectral peaks were integrated for the following ranges \(^{13}\)C: 0 – 45 ppm (alkyl C), 45 – 110 ppm (carbohydrate, alcohol and polysaccharide C), 110 – 160 ppm (aromatic C), 160 – 190 ppm (carbonyl C).

Three-dimensional fluorescence spectra of FAs were obtained using a Perkin Elmer LS50B Luminescence Spectrometer. The slit width was set at 15 nm for the excitation and emission wavelengths. The relative fluorescence intensities of the sample solutions were normalized by the intensity of a 0.1 mol dm⁻³ H₂SO₄ aqueous solution of 20 nmol dm⁻³ quinine sulfate (excitation 345 nm, emission 455 nm). The average molecular weight was determined by gel permeation chromatography, as described in a previous study.

Results and Discussion

Influences of solution conditions on the formation of precipitation

The ion-pair equilibrium between a cationic surfactant (SUR⁺) and an anionic site of FA (FA⁻) can be written as:

\[
\text{SUR}^+ + \text{FA}^- \rightleftharpoons \text{SUR}^- \cdot \text{FA}^+ \quad (3)
\]

In addition, the acid dissociation equilibrium of FA should be considered in ion-pair formation.

\[
\text{H-FA} \rightleftharpoons \text{H}^+ + \text{FA}^- \quad (4)
\]

Because H⁺-type FA (H-FA) is the dominant species in the acid supernatant, the formation of an ion-pair may not occur easily. Thus, the pH of the supernatant represents a factor in optimizing the formation of an ion-pair. Figure 2 shows the influence of solution pH and surfactant type on the percentage of FA removed. At all pHs, the percentages of FA removal for DB and BDHAC were significantly larger than those for CTAB and
DEDAB. As shown in Fig. 1, DB and BDHAC have aromatic parts in their structures. It has been known that FA can bind to aromatic compounds, such as polycyclic aromatic hydrocarbons, via hydrophobic interactions. Therefore, the higher yields of the precipitation for DB and BDHAC may be due to hydrophobic interactions between aromatic moieties in FA and surfactants as well as to ion-pair formation. In the cases of CTAB and DEDAB, the percent FA removal increased from 10% to 25% with increasing the pH of the solution. However, in the case of DB and BDHAC, the percent FA removal increased at pH values up to pH 7 and then decreased between pH 7 and 9. The decrease in FA removal at pH 9 may be due to the competition between OH− and FA− for the ammonium group in the surfactants. Therefore, the optimum pH value appears to be 7.

Other parameters (standing time and amount of surfactant) were optimized for DB and BDHAC. During a standing time of 6 - 72 h, FA removal for DB increased from 56% to 63%. However, in the case of BDHAC, an increase in FA removal with increasing standing time was not observed. Thus, we employed DB as a surfactant and 72 h for the standing time in subsequent studies. The influence of the amounts of DB used on FA removal was investigated; the addition of more than 5 mg of DB to a 2 cm^3 aliquot of supernatant was found to be optimum. In this case, the concentration of DB (6.0 mM) was above its critical micelle concentration (1.2 – 1.4 mM). Because FAs are polyvalent anionic molecules, the counter-cation having a large positive electrostatic field is required to form the ion-pair precipitation. Therefore, ion-pair formation between FA and surfactant micelle (SURmicn+) should also be considered.

\[ \text{SURmic}^n+ + n\text{FA}^- \Leftrightarrow (\text{SURmic}^n+)(\text{FA}^-)_n \]  

The precipitated ion-pair can be dissolved in aqueous HCl, which yields the surfactant chlorides (SUR-Cl) and H-FA, as shown below:

\[ \text{SUR}-\text{FA}^- + \text{HCl} \Leftrightarrow \text{SUR-Cl} + \text{H-FA} \]

Because DB is soluble in organic solvents such as CHCl3, SUR-Cl in an aqueous solution can be removed by solvent extraction. Thus, CHCl3 and CH2Cl2 were examined as organic solvents for the extraction of DB in an aqueous 0.1 mol dm^{-3} HCl solution. The extraction efficiency of DB for CHCl3 was higher than that for CH2Cl2. Three extractions with CHCl3 were sufficient to remove more than 90% of the DB from the aqueous phase. Based on these investigations, the separation and purification procedures of FA-SUR were established.

To determine whether any DB remained in the separated and purified FA-SUR or not, 1H NMR and solid-state CPMS 13C NMR spectra of FA-SUR were compared with those of DB. Figure 3 shows 1H-NMR spectra of FA-SUR and DB. In FA-SUR, broad and sharp peaks of protons corresponding to alkyl and carbohydrate appeared, as shown in a previous report. The most significant feature of the spectrum of DB was a strong peak at 2.9 ppm corresponding to methyl protons adjacent to a nitrogen atom. However, this peak was not observed in the spectrum of FA-SUR. In addition, peaks at 6.5 – 7 ppm corresponding to aromatic protons in DB were not observed in the spectrum of FA-SUR. These results support the conclusion that DB can be completely removed from FA-SUR using the procedures developed here.

**Chemical characteristics of FAs**

The chemical characteristics for FA-DAX and FA-SUR are summarized in Table 1. In elemental composition, the carbon content of FA-SUR was smaller than that of FA-DAX. However, the oxygen content and O/C atomic ratio for FA-SUR were larger than the corresponding values for FA-DAX. These results suggest that FA-SUR includes larger amounts of oxygen-containing functional groups, such as carboxylic groups, compared to the FA-DAX. As a result of these findings, the total acidity and the number of carboxylic groups in the FAs were determined. As shown in Table 1, the total acidity of FA-SUR was slightly larger than that of FA-DAX. However, the carboxylic group content of FA-SUR was similar to that of FA-DAX. In general, the subtraction of acidity due to carboxylic groups from the total acidity gives the content of phenolic hydroxyl groups. However, for acidic polysaccharides such as
alginate acid, the total acidity involves not only carboxylic groups but also hydroxyl groups on the sugar units. In particular, HSs from peat soil contain large amounts of polysaccharide-like moieties in their structures. Therefore, in the present study, subtraction of carboxylic groups from total acidity may include alcoholic hydroxyl groups and polysaccharide moieties as well as phenolic hydroxyl groups contained in the FAs.

To identify polysaccharide-like moieties in the FAs, we obtained the solid-state CPMS $^{13}$C NMR spectra. The $^{13}$C NMR spectra of FA-SUR and FA-DAX are shown in Fig. 4, and the distribution of carbon species, estimated from the $^{13}$C NMR spectra, is summarized in Table 1. The most significant feature of the spectrum of FA-SUR, compared to that of FA-DAX, was the appearance of a strong band at 70 ppm corresponding to carbohydrates or aliphatic alcohols and of a clear peak in the 100 ppm region corresponding to polysaccharides. In addition, the distribution of carbon species in the range of 45 – 110 ppm for FA-SUR was much larger than that for FA-DAX. Chemical shifts in the range of 45 – 110 ppm can include carbons corresponding to methoxy (56 ppm), carbohydrates or aliphatic alcohols (72 ppm) and polysaccharides (105 ppm). However, the carbon bands corresponding to alkyl (29 ppm) and aromatic (129 ppm) in FA-SUR were weak and broad, compared to those for FA-DAX. Therefore, the larger oxygen content in FA-SUR is due to hydroxyl groups from alcoholic and polysaccharide moieties as well as to carboxylic and phenolic hydroxyl groups.

As shown in Table 1, the distribution of aromatic carbon in FA-SUR was smaller than that in FA-DAX. The aromatic moieties in HSs are related to the levels of chromophore. Thus, spectroscopic characteristics of the FAs were determined. First, the spectroscopic parameters of FAs ($\log E_{280}$ and $\log E_{465}/E_{665}$) were compared. A larger $\log E_{280}$ value, relating to aromaticity, was observed in FA-DAX. This is consistent with the $^{13}$C NMR spectral data (Table 1), in which the distribution of aromatic carbon for FA-DAX was larger than that for FA-SUR. Another spectroscopic parameter, $\log E_{465}/E_{665}$, is known to be correlated with molecular weight. The $\log E_{465}/E_{665}$ value for FA-SUR was significantly smaller than that for FA-DAX, suggesting a lower molecular weight for FA-SUR. The average molecular weights of the FAs were measured by gel permeation chromatography; the average molecular weight for FA-SUR (11900) was slightly larger than that for FA-DAX (10100). Fluorescence spectra of FAs can also reflect both the levels of chromophore and the molecular size of the molecule. Thus, we investigated three-dimensional fluorescence spectra of the FAs. Figure 5 shows the three-dimensional excitation and emission matrices (3D-EEMs) for the FAs. Two peaks were observed for both FAs. Such patterns are consistent with findings for other FAs from soils. The EEMs and relative intensities for each peak are summarized in Table 1. The EEMs for FA-DAX were higher than those for FA-SUR, and the peak intensities for FA-SUR were lower than those for FA-DAX. It has been reported that the EEMs and fluorescence intensities increase and decrease with increasing molecular sizes of HSs, respectively. This tendency, in which the molecular size of aromatic carbon for FA-DAX was larger than that of FA-SUR, is inconsistent with the larger fluorescence intensity for FA-DAX. The fluorescence intensity can be affected by the levels of chromophore as well as by molecular size. Therefore, the weaker fluorescence intensities in FA-SUR can be attributed to the fact that the levels of chromophore in FA-SUR are lower than those in FA-DAX.

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<th>Table 1 Chemical characteristics of FA-DAX and FA-SUR</th>
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<td>Three-dimensional fluorescence spectra</td>
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Fig. 5 Three-dimensional excitation and emission matrices of FA-DAX and FA-SUR. [FAs] 2 mg dm$^{-3}$, pH 6.
Conclusion

DB was preferable for the precipitation of an ion-pair between FA and a surfactant. The structural features of FA-SUR, separated based on the formation of ion-pairs with a cationic surfactant, include larger amounts of oxygen-containing functional groups and fewer aromatic moieties, compared to FA-DAX. These results indicate that FA-SUR is more polar, in other words, more hydrophilic than FA-DAX. Such differences can be attributed to the fact that FA-SUR and FA-DAX are fractionated based on electrostatic and hydrophobic interactions, respectively. Carboxylic anions in FA appear to be major factors in ion-pair formation with a cationic surfactant. However, the carboxylic group content for FA-SUR was similar to that for FA-DAX. The solid-state CPMS $^{13}$C NMR spectra indicated that the higher levels of oxygen in FA-SUR can be attributed to alcoholic hydroxyl groups and polysaccharides. Therefore, the formation of a precipitate of FA with a cationic surfactant may be due to some interactions involving alcohols and polysaccharides as well as to electrostatic ion-pair formation with carboxylic groups.

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References