Isolation and purification of hydrophilic fulvic acids by precipitation

Syuntaro Hiradate\textsuperscript{a,}*a, Takuya Yonezawa\textsuperscript{b}, Hiroshi Takesako\textsuperscript{b}

\textsuperscript{a}Department of Biological Safety Science, National Institute for Agro-Environmental Sciences (NIAES), 3-1-3 Kan-nondai, Tsukuba, Ibaraki 305-8604, Japan
\textsuperscript{b}Department of Agriculture, Meiji University, 1-1 Higashi Mita, Tama, Kawasaki, Kanagawa 214-8571, Japan

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Abstract

Fulvic acids play an important role in the behavior of metals and hydrophobic organic chemicals in soil and water environments. The isolation and purification of the fulvic acids have been difficult to achieve, however, because these compounds are soluble in both alkaline and acidic solution, resulting in difficulty of dehydration and demineralization. We propose here a new procedure for isolating fulvic acids as precipitates. Our procedure includes pH-adjustment of the fulvic acid solution to weakly acidic to neutral pH range (4 to 7). In an Andisol, recoveries of the fulvic acids we prepared by precipitation at equilibrium pH of 5.0 were 86\% (dissolved total organic carbon basis) and 97\% (absorbance basis, 400 nm), whereas recoveries of fulvic acids adsorbed on XAD-8 resin (hydrophobic fulvic acids) were 14\% and 28\%, respectively. Recoveries of the fulvic acids were further increased in our procedure by adding Al. The mechanism forming the precipitates includes a complexation reaction of carboxylic groups of the fulvic acids with Al (ligand exchange reaction); this mechanism is identical to that for the retention of fulvic acids in many soils. Therefore, it is likely that our preparation procedure is appropriate for separating the fulvic acids stabilized in soils. Solid-state cross polarization and magic angle spinning \textsuperscript{13}C nuclear magnetic resonance spectra showed that the fulvic acids prepared by our precipitation procedure were relatively rich in O-alkyl carbons and poor in aromatic and alkyl carbons compared with those forms from the hydrophobic fulvic acids adsorbed on XAD-8 resin. A new scheme for preparing the hydrophobic and hydrophilic fulvic acids is also proposed here. The hydrophilic fulvic acids are likely to maintain high solubilities in water even after the formation of complexes with metals and may influence on their behavior.

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1. Introduction

Most sesquioxides and heavy metals have low solubilities in water, and they tend to persist in soil...
environments. These metals are, however, able to form soluble complexes with soil organic matters and move into groundwater systems. For the formation of the soluble complexes, the soil organic matters should have hydrophilic moieties, which increase the solubility in water. Dissolved soil organic matter can also enhance transport of hydrophobic organic chemicals of soils into groundwaters (Schwarzenbach et al., 2003). Therefore, hydrophilic soil organic matter has been considered to play an important role in the behavior of metals and hydrophobic organic chemicals in soil and water environments (Sparks, 2003).

To clarify the chemical properties of hydrophilic soil organic matter, research has been focused on soil fulvic acids, which have lower molecular weights, stronger acidities, and higher water-solubilities than humic acids. Preparation of the fulvic acids, however, has not been easy because they are soluble in both alkaline and acidic solution, resulting in difficulty of dehydration and demineralization. To isolate the fulvic acids as a solid phase, an adsorption procedure using a hydrophobic resin, XAD-8, was employed by Thurman and Malcolm (1981). This procedure has been widely accepted as a standard method for the preparation of fulvic acids and recommended by International Humic Substances Society (http://www.ihss.gatech.edu/). However, it should be noted that the adsorbed fraction of the fulvic acids on the XAD-8 resin strictly should be called “hydrophobic fulvic acids” and that the fraction not adsorbed by the resin, which should be more hydrophilic than the adsorbed fraction, is typically discarded. Kuwatsuka et al. (1992) reported that the amounts of non-adsorbed fraction were larger than those of the adsorbed fractions for a brown forest soil and an Ando soil.

The non-adsorbed fractions on XAD-8 resin would also persist in soil environments for a long time, as well as the adsorbed fractions. The retention mechanisms of them on soils would include coordinate bonds through metals, which are called complexation or ligand exchange reactions, resulting in polymerization and adsorption on the surfaces of soil minerals (see Fig. 1A). In our study, the complexation reaction was applied to enable the fulvic acids to be isolated as precipitates, instead of using the hydrophobic interaction by XAD-8 resin. The isolated and purified fulvic acids were characterized in terms of solid-state cross-polarization and magic angle spinning (CPMAS) $^{13}$C nuclear magnetic resonance (NMR) spectra and recoveries. Carbon-13 CPMAS NMR has been reported to be useful for characterizing soil organic matter under different management and environmental conditions (Mathers et al., 2000; Mathers and Xu, 2003; Ussiri and Johnson, 2003; Chen et al., 2004). Finally, a new scheme for preparing the hydrophobic and hydrophilic fulvic acids is proposed.

2. Materials and methods

2.1. Preparation of soil samples

Three soil samples from Japan were collected from surface horizons (A horizons). Selected characteristics are listed in Table 1.

2.2. Recoveries of fulvic acids prepared by precipitation procedure as influenced by equilibrium pH

Two kilograms of Ib-G5 soil were extracted with 16 L of 0.1 M NaOH in the presence of 3% NaCl over night. Supernatant was collected by centrifugation (10,000 $g$, 15 min). This extraction procedure was repeated three times. The supernatant (humic acid plus fulvic acid) was acidified to pH 1.0 with 4 M HCl, allowed to stand overnight, centrifuged (10,000 $g$, 15 min) to remove dispersed humic acid, and then filtered through a 0.2-$\mu$m pore-sized filter membrane (cellulose nitrate, Advantec Toyo Kaisha, Ltd, Tokyo).

A 10 mL portion of the resultant filtrate (crude fulvic acid solution) was diluted with 0.1 M NaOH and distilled water to achieve variable final pH values between 1 and 13 and a final volume of 25 mL. After incubation overnight, precipitates (fulvic acid) were removed with centrifugation (6000 $g$, 15 min). To clarify the mechanism of the precipitate formation, similar experiments were conducted in the presence of 0.05 M phosphate and 0.05 M pyrophosphate. The non-precipitated fraction of the crude fulvic acid, which remained in the supernatant, was estimated using two different parameters: absorbance at 400 nm, as determined by a spectrophotometer (U-2010, Hitachi High-Technologies Corporation, Tokyo); and concentration of dissolved total organic carbon, as determined by a total organic carbon analyzer (TOC...
Fig. 1. A scheme of reactions assumed in the present study. Al; aluminum, FA; fulvic acids, HA; humic acids, ppt; precipitates.
5000A, Shimadzu, Kyoto). Before determining the absorbance, the test solutions were brought to pH 13 by adding 1 M NaOH to a 4 mL portion of the supernatant and the final volume was adjusted to 6 mL by adding distilled water. Recoveries were calculated as percentages of precipitated fraction in the crude fulvic acid fraction.

For Ib-47 and Oki-Yo soils, a 100 g portion of the soil sample was extracted with 400 mL of 0.1 M NaOH in the presence of 3% NaCl over night. Supernatant was collected by centrifugation (10,000 \( \times g \), 15 min). The supernatant (humic acid plus fulvic acid) was acidified to pH 1.0 with 4 M HCl, allowed to stand overnight, centrifuged (10,000 \( \times g \), 15 min) to remove dispersed humic acid, and then filtered through a 0.2-\( \mu \)m pore-sized filter membrane. The filtrate (crude fulvic acid solution) was partially neutralized to pH 5.0 and allowed to stand overnight, and then the resultant precipitation (fulvic acid) was collected by centrifugation (6000 \( \times g \), 15 min). The recoveries of the fulvic acids were determined as described above.

### 2.3. Recoveries of fulvic acids prepared by adsorption procedure on XAD-8 resin (hydrophobic fulvic acids)

A glass column (22 mm i.d., 200 mm length) was filled with ca. 23 mL of Amberlite XAD-8 resin (Rohm and Haas, Philadelphia) which was washed sequentially with methanol, 0.1 M NaOH, 0.1 M HCl, and distilled water. A 100 mL portion of the crude fulvic acid solution was applied on the column, followed by washing with 100 mL of 0.1 M HCl and 100 mL of distilled water, and then the hydrophobic fulvic acid was eluted with 100 mL of 0.1 M NaOH. The recoveries of the hydrophobic fulvic acids in the crude fulvic acid solution were calculated on the basis of the absorbance at 400 nm and the concentration of dissolved total organic carbon, as described above.

### 2.4. Improvement of the recoveries of the precipitated fulvic acids by the addition of Al

A 20 mL portion of the crude fulvic acid solution, 0.1 M AlCl\(_3\) solution, and 1 M NaOH solution were mixed to achieve equilibrium pH of 5.0 and final volume of 30 mL. After incubation overnight, the supernatant was collected by centrifugation (6000 \( \times g \), 15 min), and the recoveries of the precipitated fulvic acids were determined as described above.

### 2.5. Characterization of fulvic acids by solid-state CPMAS \(^{13}\)C NMR spectra

The fulvic acids, which were prepared by the precipitation procedure and by the adsorption procedure on XAD-8 resin, were reacted with a cation exchange resin (H\(^+\)-form, Amberlite IR-120 resin, Rohm and Haas) in a beaker overnight with stirring to exchange counter cations on the fulvic acids with H\(^+\). Solid-state CPMAS \(^{13}\)C NMR spectra of the fulvic acids were recorded with a FT NMR system (Alpha 300, JEOL, Tokyo). Powdered samples of the fulvic acid, which were prepared by freeze-drying, were transferred into KEL-F spinning tubes (6 mm \( \phi \), JEOL, Tokyo). The solid-state CPMAS \(^{13}\)C NMR signals were recorded at 75.45 MHz with 6 kHz of
magic angle spinning, 1 ms of contact time, and 3 s of pulse interval. A broadening factor of 100 Hz was employed in the procedure of Fourier transformation. Standard chemical shift (0 ppm, tetramethylsilane) adjustment was performed using adamantane (29.50 ppm).

3. Results and discussion

Soil humic substances are considered to persist in surface horizons because they can form stable and strong coordinate bonds between organic ligands on the humic substances (e.g., carboxyl group) and metals in soils (e.g., Al and Fe). This is especially true for volcanic-ash derived soils or Andisols/Andosols (Wada and Higashi, 1976; Wada, 1986; Inoue, 1990; Hiradate et al., 2004), where extremely large amounts of humic substances are accumulated. The coordination reaction, frequently termed a ligand exchange reaction, is the key reaction to polymerize humus molecules with each other and to fix them on the surfaces of soil minerals (Fig. 1A). In the present study, this reaction was utilized to isolate fulvic acids as precipitates.

We assumed the following situation. When soils are treated with strong alkaline solution such as 0.1 M NaOH (pH 13), a part of the humic substances (humic and fulvic acids) is monomerized and liberated from the surfaces of soil minerals because added OH$^-$ exchanges ligands on the humic substances by the ligand exchange reactions (Fig. 1B). At this stage, part of the Al will be also dissolved as Al(OH)$_4^-$ because Al is an amphoteric electrolyte. When the supernatant is acidified to pH 1.0, Al(OH)$_4^-$ is converted to Al$^{3+}$, and the extracted humic acids are precipitated because negative charges on the humic acid molecules are neutralized by the complexation with Al$^{3+}$ or the protonation of the carboxylic group, although fulvic acids still remain in solution together with part of the Al$^{3+}$ (Fig. 1C). When the supernatant is neutralized, Al$^{3+}$ is transformed into Al hydroxides (precipitates), and the fulvic acids are also precipitated with Al hydroxides by adsorption on them (Fig. 1D) because the fulvic acids existed originally as adsorbed forms on the surfaces of soil minerals through the coordinate bonds (Fig. 1A).

To evaluate these assumptions, the recoveries of the precipitated fulvic acid in the crude fulvic acid solution prepared from Ib-G5 soil were determined as a function of equilibrium pH (Fig. 2). The fulvic acid was solubilized at pH lower than 4 and higher than 7, but it was precipitated and recovered at weakly acidic to neutral pH range between 4 and 7 (Fig. 2; A and D). The recoveries of the precipitated fulvic acid were almost constant at the pH range (4 to 7), and they were 80% to 90% on a dissolved total organic carbon basis and 90% to 100% on an absorbance basis (400 nm). The recoveries in the precipitation procedure at equilibrium pH of 5.0 (86% and 97%, respectively) were markedly higher than those resulting from the adsorption procedure on XAD-8 resin (14% and 28%, respectively) (Table 2). High recoveries in the precipitation procedure were similarly found for the paddy soil (Ib-47) and the calcareous soil (Oki-Yo), greatly exceeding those from the adsorption procedure on XAD-8 resin (Table 2). These results suggest that the precipitation procedure is very effective in isolating fulvic acids as solid phase. Such isolation of fulvic acids as precipitates is advantageous for further purification, such as dehydration and demineralization.

The formation of the fulvic acid precipitates was strongly inhibited in the presence of 0.05 M phosphate (Fig. 2; B and E) and completely inhibited in the presence of 0.05 M pyrophosphate (Fig. 2; C and F). Adsorption mechanisms of fulvic and humic acids on goethite, gibbsite, and imogolite include the ligand exchange reaction (Parfitt et al., 1977) and the sorption mechanism of phosphate on goethite (Hingston et al., 1972), on allophanic Ando soils (Nanzyo, 1987), and on clay fractions in pumice (Nanzyo, 1988). Competitive sorption of phosphate with humic substances on soils and soil constituents has been also reported (Sibanda and Young, 1986; Gerke, 1993; Hiradate and Uchida, 2004). In our study, therefore, the adsorption of fulvic acids on the Al hydroxides (Fig. 1D) would be inhibited by the competitive sorption (or complexation) of ligands, such as phosphate and pyrophosphate (Fig. 1E). It is likely that the adsorption of fulvic acids on Al hydroxides is the principal force to form fulvic acid precipitates, and that aggregation and association of the fulvic acid particles themselves by hydrophobic interactions would be less important.
These findings imply that the addition of Al might improve the recoveries of the precipitated fulvic acids if insufficient adsorbent (e.g., Al hydroxides) is present in the neutralized solution. Fig. 3 showed that the addition of AlCl₃ to the crude fulvic acid solution increased the recoveries of the fulvic acid precipitates from 0% to 20% at equilibrium pH of 5.0, depending on the soil type. In an Andisol (Ib-G5 soil), recoveries were not substantially improved by the addition of AlCl₃ (Fig. 3; A and D), implying that the crude fulvic acid solution contained excess Al to sorb fulvic acids. In paddy (Ib-47) and calcareous (Oki-Yo) soils, the recoveries were improved to some extent, but more than 30% of these fulvic acids (dissolved total organic carbon basis) remained in the solution (Fig. 3; B and C).

Table 2
Comparison of the recoveries of fulvic acids in crude fulvic acid solution isolated by the precipitation procedure proposed in the present study and by the adsorption procedure on XAD-8 resin

<table>
<thead>
<tr>
<th>Soil sample</th>
<th>Calculation basis</th>
<th>Recoveries (%)</th>
<th>Precipitation procedure⁴</th>
<th>Adsorption procedure on XAD-8 resin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ib-G5</td>
<td>TOC</td>
<td>85.9</td>
<td>14.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ABS</td>
<td>96.7</td>
<td>28.2</td>
<td></td>
</tr>
<tr>
<td>Ib-47</td>
<td>TOC</td>
<td>47.1</td>
<td>14.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ABS</td>
<td>85.5</td>
<td>33.2</td>
<td></td>
</tr>
<tr>
<td>Oki-Yo</td>
<td>TOC</td>
<td>53.2</td>
<td>18.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ABS</td>
<td>86.3</td>
<td>17.9</td>
<td></td>
</tr>
</tbody>
</table>

⁴ Precipitated at equilibrium pH of 5.0 without adding AlCl₃.

These findings imply that the addition of Al might improve the recoveries of the precipitated fulvic acids if insufficient adsorbent (e.g., Al hydroxides) is present in the neutralized solution. Fig. 3 showed that the addition of AlCl₃ to the crude fulvic acid solution increased the recoveries of the fulvic acid precipitates from 0% to 20% at equilibrium pH of 5.0, depending on the soil type. In an Andisol (Ib-G5 soil), recoveries were not substantially improved by the addition of AlCl₃ (Fig. 3; A and D), implying that the crude fulvic acid solution contained excess Al to sorb fulvic acids. In paddy (Ib-47) and calcareous (Oki-Yo) soils, the recoveries were improved to some extent, but more than 30% of these fulvic acids (dissolved total organic carbon basis) remained in the solution (Fig. 3; B and C).
C). In these soils, the non-precipitated fulvic acids would not be bound to Al through coordinate bonds, but would exist instead as other forms, such as complexes with Fe.

To characterize fulvic acids, solid-state $^{13}$C NMR spectra are commonly determined. The precipitation procedure is advantageous for such $^{13}$C NMR measurement, because it provides fulvic acids as neutral precipitates, which can be directly subjected to the solid-state NMR analyses after drying, and it provides high yields. On the other hand, in the adsorption procedure on XAD-8 resin, the hydrophobic fulvic acids are obtained in the alkaline (NaOH) solution, so they should be neutralized before freeze-drying by treatment with ion exchange resin (H$^+$-form), etc.

The fulvic acids prepared by the precipitation procedure are initially present as adsorbed form on Al hydroxides or coordinated form with Al (Al-form). These fulvic acid precipitates were easily converted to H$^+$-form by treating them with ion exchange resin (H$^+$-form) for several hours, and, as a result, soluble fulvic acids (H$^+$-form) were obtained as concentrated solution. These H$^+$-forms of the fulvic acids would be useful for liquid-state NMR measurements, functional group analyses, titration analyses, etc.

Solid-state CPMAS $^{13}$C NMR spectra of precipitated fulvic acids prepared from Ib-G5 soil were compared before (Al-form) and after (H$^+$-form) treatment with H$^+$-form of ion exchange resin (Fig. 4; A1 and A2). They were almost identical with each other.
This similarity indicates that the fulvic acids prepared by the precipitation procedure are ready for NMR analyses without conversion to the H⁺-form. Only a small shift (2 ppm shift to higher magnetic field) was observed for a resonance peak of carbonyl carbons when the Al-form (Fig. 4; A1) was converted to H⁺-form (Fig. 4; A2). Such a shift would be derived from the following reaction:

\[
R\text{COOAl} \quad (174 \text{ ppm}) \rightarrow R\text{COOH} \quad (172 \text{ ppm})
\]

The solid-state CPMAS $^{13}$C NMR spectra of fulvic acids prepared by the precipitation procedure at pH 5.0 (Fig. 4; A2, B, and C) differed greatly from those of fulvic acids prepared by the adsorption procedure on XAD-8 resin (Fig. 4; D, E, and F). The former fulvic acids were rich in $O$-alkyl (ca. 70 and 100 ppm) and carbonyl (ca. 172 ppm) carbons and poor in hydrophobic moieties such as aromatic (ca. 132 ppm) and alkyl (ca. 20 ppm) carbons. It is possible that they consist mainly of polymers of uronic acids and their derivatives. On the other hand, the latter fulvic acids were characterized by the lower contents of $O$-alkyl carbons and higher contents of carbonyl carbons and hydrophobic moieties than the former fulvic acids. Therefore, it is appropriate to define the fulvic acids prepared by adsorption as hydrophobic and the fulvic acids prepared by precipitation as hydrophilic. The hydrophobic fulvic acids have both hydrophobic and hydrophilic moieties, and so they can behave as surfactants and may influence the behavior of the soils.

Fig. 4. Solid-state CPMAS $^{13}$C NMR spectra of fulvic acids prepared by the precipitation procedure at equilibrium pH of 5.0 without the addition of AlCl$_3$ (A1, A2, B, and C) and by the adsorption procedure on XAD-8 resin (D, E, and F) from Ib-G5 (A1; Al-form, A2 and D; H⁺-form), Ib-47 (B and E; both H⁺-form), and Oki-YO (C and F; both H⁺-form) soils.
hydrophobic organic chemicals in soils by forming soluble micelles. The hydrophilic fulvic acids are composed mainly of hydrophilic moieties, and so they may influence the behavior of the metals in soils by forming soluble complexes. The hydrophilic fulvic acids have not been investigated in detail because of the difficulties in their preparation.

From the discussion above, we propose a new scheme to prepare hydrophobic and hydrophilic fulvic acids (Fig. 5). This scheme includes the standard methodologies to prepare humic acids and hydrophobic fulvic acids, but with the provision that the extractant should not contain ligands such as phosphate and pyrophosphate. In addition, the new scheme includes a recovery process for the non-adsorbed fraction on XAD-8 resin by using the precipitation procedure at weakly to neutral pH range. The resultant humic acids and hydrophobic fulvic acids have properties entirely compatible with those shown from previous studies, and additional new information is potentially obtainable from the hydrophilic fulvic acids. The validity of this scheme is to be tested further in future studies.

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**References**


