Articles

Chemical Characterization of Japanese Humic Substances Society Standard Soil Humic and Fulvic Acids by Spectroscopic and Degradative Analyses

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Abstract

The two sets of humic acids (HAs) and fulvic acids (FAs) obtained from an Umbric Andosol (Inogashira) and a Dystric Cambisol (Dando) are authorized as standard samples by the Japanese Humic Substances Society. The present paper is intended to provide information about their chemical characteristics, using ^{13}C and ^{15}N CPMAS NMR, X-ray photoelectron, and ESR spectroscopies, neutral saccharide composition analysis, and analysis of thermally assisted hydrolysis and methylation (THM) products. ^{13}C CPMAS NMR showed that the proportion of aromatic C in total C of the Inogashira Type A HAs (34.8%) was greater than that of the Dando Type P_{+} HAs (26.8%) but similar to that of the both FAs (32.9-33.5%). The proportion of *O*-alkyl C (33.6-34.1%) and the total yield of neutral monosaccharides released on acid hydrolysis (69-73 mg g^{-1}) were similar for the two HAs. The proportions of aromatic N in total N estimated by X-ray photoelectron spectroscopy (XPS) was also greater for the Inogashira HAs and the both FAs (13.2-16.6%) than for the Dando HAs (6.2%). The proportion of peptide bond N or amide N in total HA N was estimated to be 71-80% by XPS and 78-82% by ^{15}N CPMAS NMR. Second derivative ESR spectra of the two HAs showed distinct hyperfine structure including the same resonances (in different proportions for each), while only a single peak was evident in the spectra of the FAs. There was no notable difference between the two HAs in the composition of neutral monosaccharides released on acid hydrolysis. The monosaccharide composition of the HAs, in comparison with the FAs, was characterized by larger proportions of mannose and ribose, considered to originate mainly from microbes. The yield of straight-chain alkyl compounds released on THM was greater in the order, Dando HAs, Inogashira HAs, and the two FAs, corresponding to the difference in the relative content or the chemical shift of the peak maximum for alkyl C in the ^{13}C CPMAS NMR spectra. Differences between the two FAs were evident in the yields of monosaccharides and benzenepolycarboxylic acids, which were larger in the Inogashira FAs (34.2 and 16.6 mg g^{-1}) than in the Dando FAs (16.3 and 7.9 mg g^{-1}).

Introduction

In 1981, the International Humic Substances Society (IHSS) proposed a standard method to obtain purified humic acid (HA) and fulvic acid (FA) samples from solid environmental samples (International Humic Substances Society, 1981). Following this proposed method, Kuwatsuka and his co-workers in the Laboratory of Soil Science, Nagoya University, Japan, prepared, on a large scale, two sets of HAs and FAs from representative types of Japanese soils, an Umbric Andosol (Ando soil; Inogashira) and a

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Dystric Cambisol (Brown Forest soil; Dando). The aim was to investigate the applicability of the IHSS method to such definitive Japanese types of soils abundant in HAs and FAs (Kumada, 1987), by comparing the yields and chemical characteristics of HAs and FAs with those obtained by the NAGOYA method which has been used in Japan (Kuwatsuka et al., 1992; Watanabe et al., 1994). Humic substances vary among soils, not only in content but also in chemical characteristics. Consequently, the HA and FA samples from the Inogashira and Dando soils were also intended to be used as reference samples suitable for studies on the nature or function of humic substances in major soil types in Japan.

The Inogashira HAs belong to Type A (Kumada, 1987), the highest class with respect to the degree of humification. Similar HAs are commonly found in the surface layer of Ando soils and Chernozemic soils (Kumada, 1987). On the other hand, the Dando HAs are Type P_{++}, which contain the green fraction Pg (Kumada, 1987; Watanabe et al., 1996). Pg has a 4,9-dihydroxyperylene-3,10-quinone nucleus as the major chromophore, showing visible/UV absorption maxima at 615, 570, 450, and 280 nm in an alkali solution. Types P_{+}, P_{++}, and P_{+++} HAs (the number of + indicates the strength of absorptions due to Pg) are differentiated from the other types of HAs having similar A_{600}/C values, by their smaller $\log(A_{400}/A_{600})$ values. The Dando HAs have small A_{600}/C ratios, (2.42; Kuwatsuka et al., 1992; Ikeya and Watanabe, 2003), and their structural properties are near to Type Rp HAs, the lowest class with respect to the degree of humification, rather than Type B HAs, the middle class, which statistically are also common to Brown Forest soils (Ikeya and Watanabe, 2003).

The Inogashira and Dando humic substance samples were accredited as standard samples by the Japanese Humic Substances Society (JHSS) in 1991. Kuwatsuka et al. (1992) and Watanabe et al. (1994) reported their degree of humification, composition of major elements and *O*-containing functional groups, content of hexose, uronic acid, and amino acid, as well as their relative proportion of C species estimated by solution ^{13}C nuclear magnetic resonance (NMR) with inverse-gated decoupling. However, in those papers, the HA and FA samples were wholly characterized comparing different methods of preparation (NAGOYA vs IHSS). Thereafter the fluorescent spectra of the HA and FA samples were reported by Suzuki et al. (1997), and Miyajima and Mori (1996) reported the concentration of carboxyl groups in the FA samples estimated by a titration method.

In the present study, ^{13}C and ^{15}N cross polarization/magic angle spinning (CPMAS) NMR, electron spin resonance (ESR), and X-ray photoelectron spectroscopic analyses were conducted to obtain further information about chemical characteristics of the JHSS standard samples. Acid hydrolysis followed by neutral monosaccharide composition analysis and on-line gas chromatography/mass spectrometry (GC/MS) and GC of thermally assisted hydrolysis and methylation (THM) products were also performed. Similar properties obtained by different techniques are also discussed. The data will be helpful in the overall use of the JHSS samples as standards.

Materials and Methods

Samples

The HAs and FAs used were prepared from the A-horizon of Inogashira Ando soil (Fujinomiya, Shizuoka Prefecture) and Dando Brown Forest soil (Shitara, Aichi Prefecture) (IHSS method; Kuwatsuka et al., 1992). At the Inogashira site, the major vegetation was *Miscanthus sinensis*, *Sasa*, and *Torreya nucifera*, and *Quercus crispula*, *Tsuga sicboldii* Carr., and *Fagus crenata* at the Dando site.

Measurement of CPMAS NMR spectra

^{13}C CPMAS NMR spectra of the HA and FA samples were recorded at 75.6 MHz on a NMR spectrometer CMX-300 (Chemagnetic, USA) under the following conditions: spinning rate, 5 kHz; contact time, 1 msec; pulse delay time, 0.7 (HAs) or 2.0 (FAs) sec; line broadening, 100 Hz; number of accumulation, 1700-3000 times. The chemical shift was relative to tetramethylsilane (0 ppm) and adjusted with hexamethylbenzene (17.36 ppm). The spectra were divided into four regions represented by the major C species that appeared in each of them, i.e., saturated alkyl C, alkyl C substituted with heteroatom (*O*-alkyl C), aromatic C, and carbonyl C (Table 1), and the relative area of each region with respect to the total area was calculated taking spinning side bands (SSBs) into consideration (Maie et al., 2002).

^{15}N CPMAS NMR spectra of the HAs were recorded at 30.4 MHz on the same spectrometer under the following conditions: spinning rate, 4.5 kHz;

contact time, 0.7 msec; pulse delay time, 0.2 sec; line broadening, 100 Hz; number of accumulation, 160,000-260,000 times. The chemical shift was relative to nitromethane (0 ppm) and adjusted with ammonium chloride (-341.2 ppm). The spectra were divided into four regions represented by substituted pyrrole N, peptide/amide N, guanidine/aniline derivative N, and free amino N (Table 2; Knicker et al., 2000), and the N composition was obtained by the same manner as the C composition.

Measurement of N1s spectra

N1s spectra were recorded on an X-ray photoelectron spectrometer (ESCA-3300, Shimadzu, Japan) using MgKα non-monochromatic radiation at an analyzer pass energy of 32 eV, an electric current of 30 mA, and a voltage of 10 kV. A finely powdered sample (ca. 1 mg) was fixed on the surface of a metallic sample block by means of Scotch double-sided nonconducting tape. Spectra were recorded for each visible line at 0.1 or 0.05 eV per step. The time for one scan was 298 msec, and 130 to 160 scanned data were accumulated. Correction of binding energy was made relative to the C-C/C-H signal at 385.0 eV in the C1s spectra measured simultaneously. Data manipulation including curve fitting, integration, and background subtraction was accomplished on a workstation (Sun SPARC Station IPX, Shimadzu) installed with data treatment software (VISION-300, Shimadzu). The spectra were deconvoluted into three Gaussian curves with peaks at 399.0±0.1 eV for aromatic N including pyridine, imine, aniline derivatives, polycyclic aromatic amine, and NH in guanidine; 400.4±0.1 eV for peptide bond N including other amides, pyrrole, and secondary and tertiary amines; and 402.4±0.2 eV for primary amine N including protonated amines (Abe and Watanabe, 2004). Proportion of each N group in total N was estimated from the integral of Gaussian curve with respect to that of the spectra.

Measurement of solution state ESR spectra

Humic or fulvic acids (30 mg) were dissolved in 3 mL of 0.1 mol L^{-1} KOH and stirred open to the atmosphere for 8 min. The solution was then de-oxygenated by bubbling with N_2 gas for 1 min and transferred to a quartz solution cell which had previously been flushed with N_2. The cell was situated in the ESR cavity using a vacuum autosampler. Spectra were recorded on a ECS 106 spectrometer (Bruker, Germany) operating at ca. 9.5 GHz frequency at 22°C with the following operating conditions: microwave power, 1.008 mW; modulation amplitude, 0.213 G; sweep width for first derivative spectra, 10 G; sweep width for second derivative spectra, 5 G; center field, 3462.65 G; modulation frequency, 100 kHz; conversion time, 20.48 msec; time constant, 10.24 msec. The first derivative spectra were the computer average of 10 scans and the second derivative spectra of 20 scans.

Relative free-radical concentration in the samples was estimated based on the double integral of the first derivative spectra after 30 min in 0.1 mol L^{-1} KOH. That in the FAs was determined also from solid ESR spectra according to Watanabe et al. (submitted for publication).

Table 1. Composition of C species in humic and fulvic acids estimated by CPMAS NMR method (%).

Fraction	Soil	Carbonyl C (160-190 ppm)	Aromatic C (110-160 ppm)	*O*-alkyl C (45-110 ppm)	Alkyl C (0-45 ppm)
HAs	Inogashira	14.8	34.8	33.6	16.8
	Dando	14.8	26.8	34.1	24.3
FAs	Inogashira	18.6	32.9	30.6	18.0
	Dando	18.9	33.5	30.0	17.5

Table 2. Composition of N species in humic acids estimated by CPMAS NMR method (%).

Soil	Substituted pyrrole N (-145 to -220 ppm)	Peptide/amide N (-220 to -285 ppm)	Guanidine/aniline derivative N (-285 to -325 ppm)	Free amino N (-325 to -375 ppm)
Inogashira	12	78	4	6
Dando	12	82	3	3

Measurement of monosaccharide composition

Samples (50 mg HAs or 100 mg FAs) were wetted in 2 mL of 12 mol L^{-1} H_2SO_4 for 16 h at room temperature, then diluted with 46 mL H_2O, and heated at 105°C for 8 h. The internal standard, containing with 80 μg myo-inositol, was added to the hydrolysate and reacted with 50 mg $NaBH_4$ overnight at room temperature. The reduced sample was acetylated with 7 mL acetic anhydride by heating at 105°C for 8 h and extracted with diethylether. An aliquot of the diethyl ether solution was introduced to a gas chromatograph HRGC 5160 (Carlo Erba, Italy) with a flame ionization detector (FID). An SP-2380 (Supelco, USA) fused silica column (30 m length × 0.25 mm i.d. × 0.20 μm film thickness) was used with helium (He) as the carrier gas. Column temperature was held constant at 150°C for 5 min, and increased to 250°C at a rate of 7.5°C min^{-1}, holding the final temperature for 15 min. A split ratio of 40:1 was used. Acid hydrolysis followed by determination of neutral monosaccharides was conducted in duplicate.

THM-GC/MS and -GC with tetramethylammonium hydroxide (TMAH)

A sample (150 μg) was weighed in a platinum cup, and 4 μL of dichloromethane solution containing 100 ng of nonadecanoic acid methyl ester (ME) and 4 μL of methanol solution containing 0.87 mg TMAH were added as an internal standard and as methylation reagent, respectively. The cup was introduced to a vertical microfurnance pyrolyzer PYR-4A (Shimadzu) connected to a gas chromatograph/mass spectrometer, GC-17A/QP5050 (Shimadzu) or a PY-2020D (Frontier Lab, Japan) connected to a gas chromatograph HP6890 (Agilent, USA) with a FID. The sample was flash-pyrolysed at 500°C, and the degradation products were immediately carried in He to, and separated on, a metal capillary column, Ultra ALLOY-PY1 (Frontier Lab; 30 m length × 0.25 mm i.d. × 0.25 μm film thickness). The column temperature was programmed from 50 to 300°C at 6°C min^{-1}, holding the final temperature for 15 min. Peak assignment was conducted by comparing the resultant mass spectra with the WILEY mass spectral library (USA). Yields of the assigned compounds were calculated by GC-FID using the internal standard method. Peaks not showing mass spectra as a single compound were regarded as 'not detected' even if those having the same retention time could be assigned in other samples. Quantitative analysis was conducted in triplicate. Experimental error for alkyl compounds, phenol compounds, and benzenepolycarboxylic acids was <10%, <18%, and <8%, respectively.

Results and Discussion

^{13}C CPMAS NMR spectra

The ^{13}C CPMAS NMR spectra (Fig. 1) exhibited typical differences which have often been observed

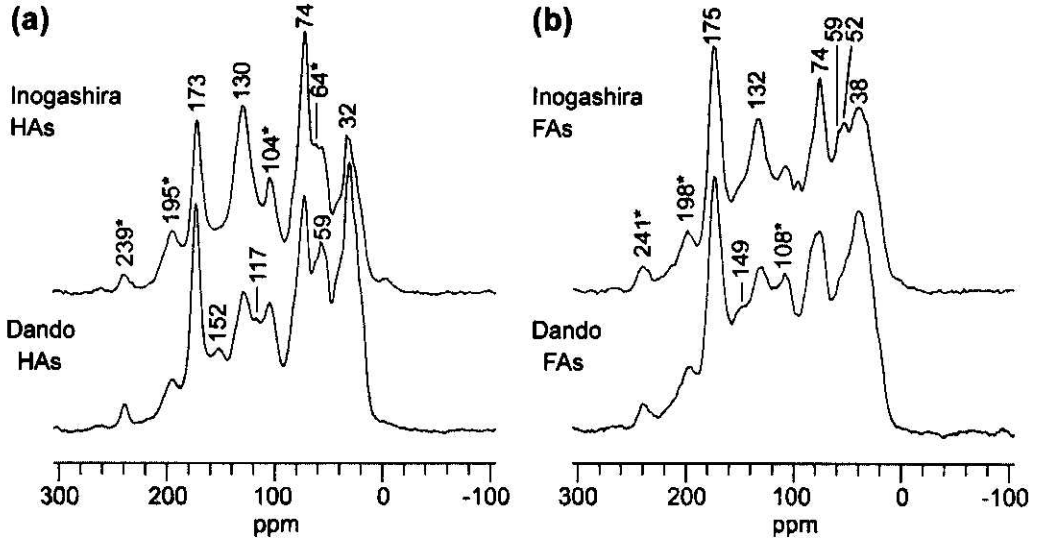

Figure 1. ^{13}C CPMAS NMR spectra of (a) humic and (b) fulvic acids. *, Spinning side band (SSB) or including SSB.

between the Type A HAs and Type P or other HAs with a lower degree of humification (Ikeya et al., 2004; Maie et al., 2002) and between HAs and FAs; i.e., the higher and lower proportions of aromatic and alkyl C in total C for the Inogashira HAs than for the Dando HAs, respectively, and the higher proportion of carbonyl C for the two FAs than for the two HAs (Table 1). For soil HAs, there is a positive correlation between the proportion of aromatic C in total C and A_{600}/C (Ikeya et al., 2004). However, the proportion of aromatic C for the Inogashira HAs, 34.8%, was lower than that expected from their high degree of humification, while the proportion of *O*-alkyl C was as high as that for the Dando HAs, 33.6-34.1%. Thus, the Inogashira HAs are considered to consist of molecules, both very high and low in aromaticity, with the former having polycondensed aromatic cores or containing multi-conjugated systems to demonstrate the high degree of humification.

Another notable difference between the HAs and FAs was the peak maximum for alkyl C, which appeared at 32 and 33 ppm in the Inogashira and Dando HAs, but at 38 ppm in the two FAs. This suggests a greater abundance of straight-chained alkyl components in the HAs, whereas a more branched variety in the FAs.

Contrary to the HAs, the compositions of C species in the Inogashira and Dando FAs were similar. Table 3 compares carbonyl C or carboxyl group concentration estimated by different methods. As the carbonyl C concentrations estimated by ^{13}C NMR include amide C=O (Monteil-Rivera et al., 2000), they should be larger than the carboxyl group concentrations estimated by titration. Based on the total N content (Watanabe et al., 1994) and the proportion of peptide bond N in total N estimated by X-ray photoelectron spectroscopy (XPS; Table 4; shown later), amide C=O concentration was estimated to be ca. 0.96 and 0.39 mmol g^{-1} for the Inogashira and Dando FAs, respectively. The sum of amide C=O with carboxyl group concentration obtained by titration with a calcium acetate solution, 10.8 and 8.1 mmol g^{-1}, was considerably close to the carbonyl C concentration estimated by solution ^{13}C NMR, although the co-occurrence of aldehyde and ketone in the carbonyl C and that of indole, pyrrole, secondary and tertiary amines in the peptide bond N could not be excluded. The proportion of carbonyl C estimated by the CPMAS method was probably underestimated due to the low sensitivity to C not substituted with H (Preston, 1996; Keeler and Maciel, 2003). As the other three datasets showed a larger concentration of carboxyl groups or carbonyl C in the Inogashira FAs than in the Dando FAs, the carbonyl C concentration based on ^{13}C CPMAS NMR might be underestimated significantly for the Inogashira FAs.

N1s and ^{15}N CPMAS NMR spectra

The N1s spectra of the samples are shown in Fig. 2. The contents of three groups of aromatic N, peptide bond N, and primary amine N in total N estimated by XPS (Table 4) were 13.2, 70.7, and 16.2% for the

Table 3. Comparison of content of carboxyl group or carbonyl C in fulvic acids estimated by different method

Soil	-- ^{13}C NMR (carbonyl C; mmol g^{-1}*) --		---- Chemical analysis (carboxyl group; mmol g^{-1}) ----	
	CPMAS	Inverse-gated decoupling**	Titration with CH_3COOCa**	Titration with NaOH†
Inogashira	6.70	11.0	9.81	7.2
Dando	7.49	8.79	7.72	6.4

*(Percentage of carbonyl C in total C) × total C content (Watanabe et al., 1994).
** Cited from Watanabe et al. (1994). † 0.01 mol L^{-1} NaOH + 0.01 mol L^{-1} NaCl (Miyajima and Mori, 1996).

Table 4. Composition of N species in humic and fulvic acids estimated by XPS method.

Fraction	Soil	Aromatic N (%)	Peptide bond N (%)	Primary amine N (%)
HAs	Inogashira	13.2	70.7	16.2
	Dando	6.2	80.3	13.6
FAs	Inogashira	13.2	77.7	9.1
	Dando	16.6	70.2	13.2

Inogashira HAs, and 6.2, 80.3, and 13.6% for the Dando HAs, respectively. The higher and lower percentages of aromatic and peptide bond N for the Inogashira HAs than for the Dando HAs agreed to the trend observed for other pairs of HAs with different degrees of humification, and the values relative to A_{600}/C were similar to other soil HAs (Abe and Watanabe, 2004).

Peptide bond N was also the major N functional group in the FAs, which accounted for 70.2 (Dando) and 77.7% (Inogashira) of total N (Table 4). The larger value for the Inogashira FAs than for the Dando FAs was similar to the previous results of total N and amino acid contents (Watanabe et al., 1994). The proportion of aromatic N, 13.2 and 16.6%, was similar to that for the Inogashira Type A HAs and larger than those for the Dando and other HAs with low and medium degrees of humification (Abe and Watanabe, 2004).

The ^{15}N CPMAS NMR spectra of the HAs and the composition of N species estimated from them, are shown in Fig. 3 and Table 2 respectively. The interpretation of ^{15}N CPMAS NMR spectra is more difficult than that of ^{13}C CPMAS NMR spectra, due to the small N content, diminutive atom-^{15}N%, and much smaller content of other N species than peptide/amide N. The proportions of peptide/amide N, substituted pyrrole N, guanidine/aniline derivative N, and free amino N were 78-82%, 12%, 3-4%, and 3-6%, respectively. Pyridine N (-25 to -90 ppm) was not evident above background noise. Although any differences between the Inogashira and Dando HAs were smaller compared with the XPS results, the identity of peptide/amide N as the major N species, in a larger proportion for the Dando HAs than for the Inogashira HAs, was similar. If the resonance in the free amino N region is assumed to originate mainly from the terminal amino groups of peptide and proteinaceous materials, the larger average molecular weight of these in the Dando HAs, compared with the Inogashira HAs is suggested by the lesser ratio of free amino N to peptide/amide N in the Dando HAs.

ESR spectra

The second derivative ESR spectra of the HAs and FAs in 0.1 mol L^{-1} KOH are shown in Fig. 4. Although free-radical species in humic substances have been suggested to include semiquinones, methoxybenzenes, and heterocyclics (Senesi, 1990), the components contributing to hyperfine structure observed in their solution state ESR spectra have not been identified (Cheshire and Senesi, 1998; Cheshire and McPhail, 1996). Hence, here the comparison of ESR spectra among the samples is conducted. The ESR spectrum of the Inogashira HAs was very similar in pattern to those of other Ando soil HAs (Watanabe et al., submitted for publication). The Dando HAs contained the same resonances as the Inogashira HAs, but in different proportions. The hyperfine peaks in the ESR spectrum of the Inogashira FAs were weak, and the structure was simple in both the FAs compared with the HAs. The second derivative spectra of the Dando HAs and the two FAs reflected a single sharp resonance of peak-to-peak width ca.

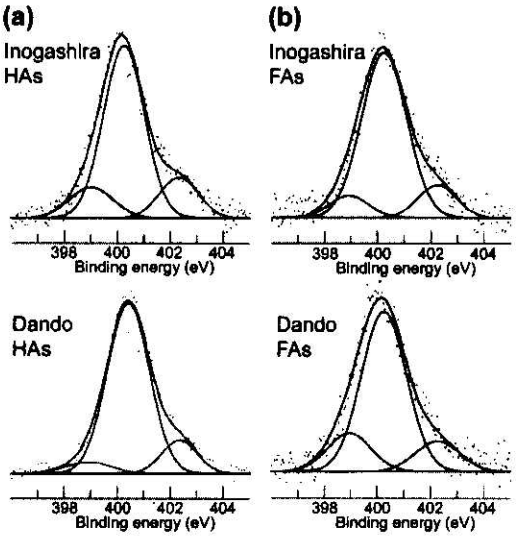

Figure 2. N1s spectra of (a) humic and (b) fulvic acids.

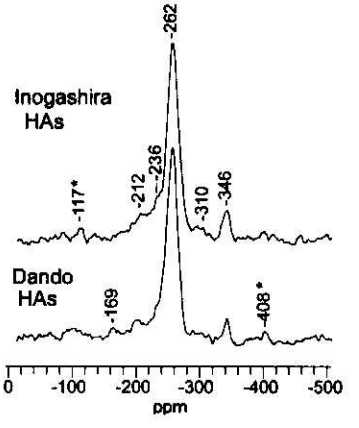

Figure 3. ^{15}N CPMAS NMR spectra of humic acids.
*: Spinning side bands of peptide/amide N signal.

0.05 mT that was superimposed on a broad background signal in their first derivative spectra (data not shown). There is a possibility that a peak with similar characteristics to the ESR spectrum of the Dando FAs affects that of the Dando HAs.

After 30 min in 0.1 mol L^{-1} KOH, the relative concentration of free-radicals determined was 1.00, 0.69, 0.30, and 0.30 for the Inogashira HAs, Dando HAs, Inogashira FAs, and Dando FAs, respectively. For solid samples, the corresponding values were 1.00, 0.26 (Watanabe et al., submitted for publication), 0.25, and 0.09. For the Dando HAs and FAs, the difference in free-radical concentration compared with the Inogashira HAs was smaller in 0.1 mol L^{-1} KOH than in the solid state. Hence, free-radicals were more stable in the Inogashira samples than in the Dando samples in the fully-protonated form.

Monosaccharide composition

Although neutral saccharides are commonly detected in acid hydrolysates of soil humic substances (Malcolm and McCarthy, 1991; Watanabe and Kuwatsuka, 1992; Watanabe et al., 1994), the data of monosaccharide composition is limited in number (Ogner, 1980; Cheshire et al., 1992; Clapp and Hayes, 1999). The total yield of eight neutral monosaccharides varied between 69.1-72.6 mg g^{-1} for the two HAs and 16.6-34.2 mg g^{-1} for the two FAs (Table 5). The sum of hexoses in the Dando HAs, 40.2 mg g^{-1}, was comparable with the hexose content estimated by colorimetry, 41.5 mg g^{-1} (Watanabe et al., 1994). For the other samples, the present result was 1.3 to 3.9 times greater than the hexose content estimated by colorimetry, although direct comparison of the method of determination is difficult because of differences in hydrolysis conditions. Tsutsuki and Kuwatsuka (1979) reported that the hexose content in soil HAs decreased with the increase in the degree of humification. A similar relation was not observed between the Inogashira and Dando HAs due to the large carbohydrate content of the Inogashira HAs (also indicated by ^{13}C CPMAS NMR; Fig. 1 and Table 1).

The monosaccharide composition (Table 5) was similar between the two HAs. Glucose accounted for the greatest proportion (20.6-22.8%), followed by mannose (18.7-19.1%) and xylose (18.2-18.5%). In the Dando FAs, glucose accounted for the greatest in proportion (24.9%), and the contribution of arabinose

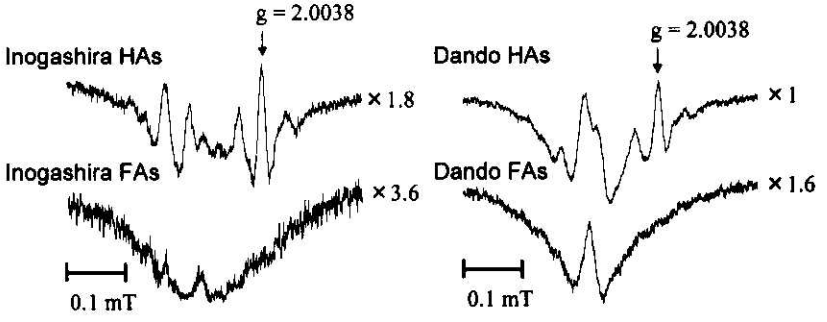

Figure 4. Second derivatives of ESR spectra of humic and fulvic acids in 0.1 mol L^{-1} KOH.

Table 5. Monosaccharide contents in humic and fulvic acids (mg g^{-1})*.

Fraction	Soil	Glucose	Galactose	Mannose	Xylose	Arabinose	Ribose	Fucose	Rhamnose	Total
HAs	Inogashira	17.7	11.3	14.5	11.8	7.6	1.6	2.0	6.1	72.6
		(22.8)**	(14.5)	(18.7)	(18.2)	(11.8)	(2.5)	(2.8)	(8.7)	
	Dando	15.3	10.7	14.2	11.4	7.3	1.5	2.3	6.5	69.1
		(20.6)	(14.5)	(19.1)	(18.5)	(11.8)	(2.5)	(3.4)	(9.6)	
FAs	Inogashira	7.5	4.9	5.7	9.3	3.8	0.1	1.1	1.8	34.2
		(20.1)	(13.3)	(15.2)	(30.0)	(12.4)	(0.3)	(3.3)	(5.5)	
	Dando	4.4	2.7	2.7	2.5	2.3	0.1	0.4	1.5	16.6
		(24.9)	(15.0)	(15.4)	(16.8)	(15.6)	(0.7)	(2.6)	(9.1)	

*Ash free basis. **Mol% in total monosaccharides.

(15.6%) was greater compared with the two HAs, 11.8%. In the Inogashira FAs, xylose occupied the greatest proportion (30.0%). In the soil, arabinose and xylose are considered to derive mainly from higher plant (Murayama, 1988). The amounts of mannose (15.2-15.4%) and ribose (0.3-0.7%) in the FAs were smaller than those in the HAs in both soils. These sugars are known to originate from microorganisms (Murayama, 1988). Hence, the present results suggested that the HAs contained microbially-synthesized sugars in a greater proportion than the FAs in both soils.

Thermally assisted hydrolysis and methylation products

Figure 5 shows the FID chromatograms of the HAs and FAs obtained by THM with TMAH. Assignment of peaks and the yields of assigned compounds are presented in Table 6. Total yields of the assigned compounds were 6.8±1.8 and 14.5±0.4 mg g^{-1} for the Inogashira and Dando HAs, respectively; those for the Inogashira and Dando FAs were 31.7±4.1 and 21.9±2.1 mg g^{-1}.

Saturated fatty acids methyl esters (FAMEs) ranging from C15 to C30 were found in the two HAs. The sum of the yields of FAMEs was greater in the Dando HAs than in the Inogashira HAs. Since a major difference between the two HAs was evident in the yields of C20 to C26 FAMEs, the lower yield of FAMEs in the Inogashira HAs can be attributed largely to lack of FAMEs derived from higher plants (Kolattukudy, 1980). For the FAs, only 3 FAMEs of C16, C18, and C24 were detected. The C16 and C18 FAMEs could derive both from microbes and plants. Other alkyl compounds, C16 to C26 dicarboxylic acids MEs (DAMEs) and C16 to C26 monomethoxy FAMEs except for C22 monomethoxy FAME, were detected only in the HAs. Yields of many of them were also greater in the Dando HAs than in the Inogashira HAs. The lower yield of alkyl compounds in the HAs with a higher degree of humification was also observed by Ikeya et al. (2004) in curie-point pyrolysis GC/MS with TMAH. They also found a significant positive correlation between the sum of the yields of aliphatic compounds and the proportion of alkyl C in total C estimated by ^{13}C CPMAS NMR for 18 soil HAs. Such relationship did not occur when the FAs were included (Tables 1 and 6). It is consistent with the interpretation of ^{13}C CPMAS NMR spectra, i.e., more abundance of branched alkyl components in the FAs than in the HAs (Fig. 1), although the total yield of alkyl compounds by THM-GC was much smaller than the total alkyl C content.

Four of five phenol compounds assigned were related to lignin and the remaining one, 4-methoxybenzoic acid ME, could derive both from lignin and other origins (Hedges et al., 1982). Irrespective of an inclusion of 4-methoxybenzoic acid ME, the sum of the yields of phenol compounds was larger in the FAs than in the HAs, but similar between the two FAs. The larger yield of phenol compounds from the Dando HAs than from the Inogashira HAs were identified with more distinct resonances of methoxyl (59 ppm) and *O*-aryl (152 ppm) C in the ^{13}C CPMAS NMR spectra for the Dando HAs (Fig. 1).

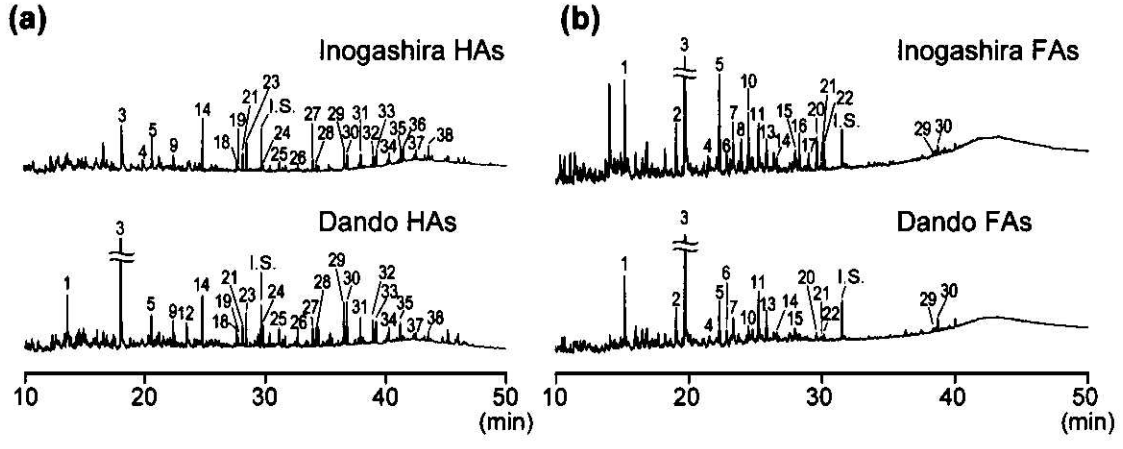

Figure 5. FID chromatograms of (a) humic and (b) fulvic acids obtained by THM-GC with tetramethylammonium hydroxide. Numbers are given to assigned peaks and correspond to those in Table 6. I.S., Internal Standard.

Table 6. Assignment and yields of compounds obtained by thermally assisted hydrolysis and methylation with tetramethylammonium hydroxide.

Peak No	Name of compounds	HAs		FAs	
		Inogashira (mg g^{-1})*	Dando (mg g^{-1})	Inogashira (mg g^{-1})	Dando (mg g^{-1})
Alkyl compounds					
2	C9 DAME**	N.D.†	N.D.	1.75	1.08
9	C15 FAME††	0.19	0.43	N.D.	N.D.
14	C16 FAME	0.69	0.83	0.25	0.18
18	C18:1 FAME (1)‡	0.16	0.32	N.D.	N.D.
19	C18:1 FAME (2)	0.09	0.22	N.D.	N.D.
21	C18 FAME	0.24	0.36	0.17	0.07
23	C16 monomethoxy FAME	0.40	0.56	N.D.	N.D.
24	C16 DAME	0.07	0.46	N.D.	N.D.
25	C20 FAME	0.12	0.24	N.D.	N.D.
26	C18 DAME	0.14	0.37	N.D.	N.D.
27	C22 FAME	0.14	0.30	N.D.	N.D.
28	C20 monomethoxy FAME	0.09	0.22	N.D.	N.D.
29	C24 FAME	0.25	0.66	0.09	0.07
30	C22 monomethoxy FAME	0.23	0.67	0.20	0.29
31	C22 DAME	0.23	0.47	N.D.	N.D.
32	C26 FAME	0.14	0.39	N.D.	N.D.
33	C24 monomethoxy FAME	0.22	0.35	N.D.	N.D.
34	C24 DAME	0.17	0.24	N.D.	N.D.
35	C28 FAME	0.19	0.26	N.D.	N.D.
36	C26 monomethoxy FAME	0.16	N.D.	N.D.	N.D.
37	C26 DAME	0.17	0.19	N.D.	N.D.
38	C30 FAME	0.22	0.15	N.D.	N.D.
Phenol compounds					
1	4-Methoxybenzoic acid ME‡‡	N.D.	1.83	2.31	2.19
3	3,4-Dimethoxybenzoic acid ME	1.21	3.67	8.19	8.18
4	3-(4-Methoxyphenyl)-2-propenoic acid ME	0.23	N.D.	0.68	0.20
5	3,4,5-Trimethoxybenzoic acid ME	0.95	1.05	3.68	1.79
12	3-(3,4-Dimethoxyphenyl)-2- propenoic acid ME	0.09	0.23	N.D.	N.D.
Benzenepolycarboxylic acids					
6	Monomethoxybenzenedicarboxylic acid diME (1)	N.D.	N.D.	0.98	0.71
7	Monomethoxybenzenedicarboxylic acid diME (2)	N.D.	N.D.	1.35	1.13
8	Benzenetricarboxylic acid triME (1)	N.D.	N.D.	1.73	N.D.
10	Benzenetricarboxylic acid triME (2)	N.D.	N.D.	2.03	1.05
11	Mixture ¶	N.D.	N.D.	2.21	1.92
13	Dimethoxybenzenedicarboxylic acid diME (2)	N.D.	N.D.	1.44	1.28
15	Methoxybenzenetricarboxylic acid triME (1)	N.D.	N.D.	0.76	0.57
16	Methoxybenzenetricarboxylic acid triME (2)	N.D.	N.D.	0.55	0.36
17	Benzenetetracarboxylic acid tetraME (1)	N.D.	N.D.	0.66	N.D.
20	Benzenetetracarboxylic acid tetraME (2)	N.D.	N.D.	1.61	0.52
22	Benzenetetracarboxylic acid tetraME (3)	N.D.	N.D.	1.06	0.31
Sum of *alkyl compounds*		4.32	7.70	2.46	1.69
Sum of *phenol compounds*		2.47	6.77	14.86	12.36
(Sum of lignin-related compounds) ¶¶		(2.47)	(4.95)	(12.55)	(10.17)
Sum of *benzenepolycarboxylic acids*		N.D.	N.D.	14.41	7.87
Total		6.80	14.48	31.72	21.91

* Ash free basis. ** Dicarboxylic acid dimethyl ester. † Not determined due to a lower yield than approximately 0.06 mg g^{-1} or incomplete isolation. †† Fatty acid methyl ester. ‡ Same compounds followed by different number in parenthesis are isomers. ‡‡ Methylester. ¶ Mixture of dimethoxybenzenedicarboxylic acid diME (1) and benzenetricarboxylic acid triME (3).
¶¶ Sum of peak nos. 3, 4, 5, and 12.

The 3,4,5-trimethoxybenzoic acid ME to 3,4-dimethoxybenzoic acid ME ratio was larger in the Inogashira HAs (0.79) and FAs (0.45), compared with the Dando HAs (0.29) and FAs (0.22). The sum of the yields of cinnamyl (phenylpropenoid) compounds (peak nos. 4 and 12) was also larger in the Inogashira samples. These differences suggest a more-significant contribution from nonwoody angiosperm tissues as an origin of phenol compounds in the Inogashira samples (Hedges and Mann, 1979), which could be attributed to *Miscanthus sinensis*, major vegetation at the soil sampling site. Hatcher and Minard (1995) observed the preferential generation of acid type monomers from wood and model compounds in the course of off-line THM with TMAH at 250°C for 30 min. Lignin-related compounds yielded from several soils at the same conditions also showed much higher acid to aldehyde ratio compared with those obtained by the CuO oxidation method (Hatcher et al., 1995). Although their reaction conditions differed from ours, similar reactions might be related to the present results, in which only the acid type of lignin-related compounds were detected.

Benzene di-, tri-, and tetra-carboxylic acids MEs were detected only in the FAs. However, these results did not indicate the absence of such components in the HAs. Actually, 1,3-, 1,4-, and 3,4-benzenedicarboxylic acids MEs, accompanied by 3,4,5-, 1,2,4-, and 1,3,5-benzenetricarboxylic acids MEs were detected in numerous soil HAs by curie-point pyrolysis GC/MS with TMAH, in which a larger amount of HAs was subjected to analysis (Ikeya et al., 2004). Thus, the present results should be interpreted whereby the contents of benzenepolycarboxylic acids are smaller in HAs than in FAs. The sum of the yields of benzenepolycarboxylic acids MEs also differed between the two FAs, owing to larger yields of benzene tri- and tetra-carboxylic acids MEs in the Inogashira FAs than in the Dando FAs.

Smaller yields of alkyl and phenol compounds from the Inogashira HAs than from the Dando HAs, corresponded to their differences in degree of humification. The smaller proportion of peptide/amide N in total N and lesser total N content (Watanabe et al., 1994) also suggest that there is a lesser content of peptide and protaineous components in the Inogashira HAs than in the Dando HAs. The similar content of neutral saccharides within them (Table 5), thus, seemed to be an exception; some carbohydrate components might be protected from microbial attack by the highly-humified matrix of the Inogashira HAs.

The yield of plant-derived FAMEs was larger in the HAs than in the FAs, while the yield of lignin-related compounds (Table 6) and the proportion of plant-derived sugars in total neutral saccharides (Table 5) were larger in the FAs than in the HAs. The inconsistency in the major origin of HAs and FAs among the components probably depends on their solubilities at low pH in the final extraction solution. Thus, the comparison of chemical composition of HAs and FAs between soils or other environments should be done separately for each of them.

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References

Abe, T. and Watanabe, A. (2004) X-ray photoelectron spectroscopy of nitrogen functional groups in soil humic acids. *Soil Sci.*, **169**, 35-43.

Cheshire, M.V. and McPhail, D.B. (1996) Hyperfine splitting in the electron spin resonance solution spectra of humic substances. *Eur. J. Soil Sci.*, **47**, 205-213.

Cheshire, M.V. and Senesi, N. (1998) Electron spin resonance spectroscopy of organic and mineral soil particles. *In* Structure and Surface Reactions of Soil Particles, Eds. P.M. Huang, N. Senesi, and J. Buffle, p. 325-373, John Wiley & Sons, Chichester.

Cheshire, M.V., Russell, J.D., Fraser, A.R., Bracewell, J.M., Robertson, G.W., Benzing-Purdie, L.M., Ratcliffe, C.I., Ripmeester, J.A., and Goodman, B.A. (1992) Nature of soil carbohydrate and its association with soil humic substances. *J. Soil Sci.*, **43**, 359-373.

Clapp, C.E. and Hayes, M.H.B. (1999) Characterization of humic substances isolated from clay- and silt-sized fractions of a corn residue-amended agricultural soil. *Soil Sci.*, **164**, 899-913.

Hatcher, P.G. and Minard, R.D. (1995) Comment on the origin of benzenecarboxylic acids in pyrolysis methylation studies. *Org. Geochem.*, **23**, 991-994.

Hatcher, P.G., Nanny, M.A., Minard, R.D., Dible, S.D., and Carson, D.M. (1995) Comparison of two thermochemolytic methods for the analysis of lignin in decomposing gymnosperm wood: the CuO oxidation method and the method of thermochemolysis with tetramethylammonium hydroxide (TMAH). *Org. Geochem.*, **23**, 881-888.

Hedges, J.I. and Mann, D.C. (1979) The characterization of plant tissues by their lignin oxidation products. *Geochim. Cosmochim. Acta*, **43**, 1803- 1809.

Hedges, J.I., Ertel, J.R., and Leopold, E.B. (1982) Lignin geochemistry of a late quaternary sediment core from Lake Washington. *Geochim. Cosmochim. Acta*, **46**, 1869-1877.

Ikeya, K. and Watanabe, A. (2003) Direct expression of an index for the degree of humification of humic acids using organic carbon concentration. *Soil Sci. Plant Nutr.*, **49**, 47-53.

Ikeya, K., Yamamoto, S., and Watanabe, A. (2004) Semiquantitative GC/MS analysis of thermochemolysis products from soil humic acids with various degree of humification. *Org. Geochem.*, **35**, 583-594.

International Humic Substances Society (1981) Outline of extraction procedures. distributed to the members of International Humic Substances Society.

Keeler, C. and Maciel, G.E. (2003) Quantitation in the solid-state ^{13}C NMR analysis of soil and organic soil fractions. *Anal. Chem.*, **75**, 2421-2432.

Knicker, H., Schmidt, M.W.I., and Kgel-Knabner, I. (2000) Nature of organic nitrogen in fine particle size separates of sandy soils of highly industrialized areas as revealed by NMR spectroscopy. *Soil Biol. Biochem.*, **32**, 241-252.

Kolattukudy, P.E. (1980) Biopolyester membranes of plants: cutin and suberin. *Science*, **208**, 990-1000.

Kumada, K. (1987) Chemistry of Soil Organic Matter, 241pp., Elsevier, Amsterdam.

Kuwatsuka, S., Watanabe, A., Itoh, K., and Arai, S. (1992) Comparison of two methods of preparation of humic and fulvic acids, IHSS method and NAGOYA method. *Soil Sci. Plant Nutr.*, **38**, 23-30.

Maie, N., Watanabe, A., Hayamizu, K., and Kimura, M. (2002) Comparison of chemical characteristics of Type A humic acids extracted from subsoils of paddy fields and surface ando soils. *Geoderma*, **106**, 1-19.

Malcolm, R. L. and McCarthy, P. (1991) The individuality of humic substances in diverse environments. *In* Advances in Soil Organic Matter Research: The Impact on Agriculture & the Environments, Ed. W.S. Wilson, p. 23-34, The Royal Soc. Chem., Cambridge.

Miyajima, T. and Mori, M. (1996) Complexation equilibria of humic substances in aqueous solution. *Bunseki Kagaku*, **45**, 369-399 (in Japanese with English summary).

Monteil-Rivera, F., Brouwer, E.B., Masset, S., Deslandes, Y., and Dumonceau, J. (2000) Combination of X-ray photoelectron and solid-state ^{13}C nuclear magnetic resonance spectroscopy in the structural characterization of humic acids. *Anal. Chim Acta*, **424**, 243-255.

Murayama, S. (1988) Microbial synthesis of saccharides in soils incubated with ^{13}C-labelled glucose. *Soil Biol. Biochem.*, **20**, 193-199.

Ogner, G. (1980) Analysis of the carbohydrates of fulvic and humic acids as their partially methylated alditol acetates. *Geoderma*, **23**, 1-10.

Preston, C.M. (1996) Applications of NMR to soil organic matter analysis: History and prospects. *Soil Sci.*, **161**, 144-166.

Senesi, N. (1990) Application of electron spin resonance (ESR) spectroscopy in soil chemistry. *In* Advances in Soil Science. Vol. 14, Ed. B.A. Stewart, p.77-130, Springer-Verlag, New York.

Suzuki, Y., Nagao, S., Nakaguchi, Y., Matsunaga, T., Muraoka, S., and Hiraki, K. (1997) Spectroscopic properties of fluorescent substances in natural waters (1). *Chikyukagaku*, **31**, 171-180 (In Japanese with English summary).

Tsutsuki, K. and Kuwatsuka, S. (1979) Chemical studies on soil humic acids. IV. Amino acid, phenol, and sugar composition in the acid hydrolysable fraction of humic acids. *Soil Sci. Plant Nutr.*, **25**, 29-38.

Watanabe, A., Fujimori, H., Nagai, Y., Miyajima, T., and Kuwatsuka, S. (1996) Analysis of the green fraction of humic acids. *Eur. J. Soil Sci.*, **47**, 197-204

Watanabe, A. and Kuwatsuka, S. (1992) Ethanol-soluble and insoluble fractions of humic substances in soil fulvic acids. *Soil Sci. Plant Nutr.*, **38**, 391-399.

Watanabe, A., Itoh, K., Arai, S., and Kuwatsuka, S. (1994) Comparison of the composition of humic and fulvic acids prepared by the IHSS method and NAGOYA method. *Soil Sci. Plant Nutr.*, **40**, 601-608.